CANADIAN JOURNAL OF RESEARCH

VOLUME 19 JULY, 1941 NUMBER 7

CONTENTS

SECTION C.—BOTANICAL SCIENCES

Page

The Reaction of *Fragaria virginiana* to the Virus of Yellow-edge—*A. A. Hildebrand* - - - - - - - - - 225

Varietal Differences in Barleys and Malts. XI. Simultaneous Relations Between Malt Extract and Two or More Barley Properties—*H. R. Sallans, W. O. S. Meredith, and J. A. Anderson* - - - - - - - - - - - - - 234

Studies on Film-forming Yeasts. I. Media and Methods—*V. E. Graham and E. G. Hastings* - - - - - - - - 251

Vegetative Propagation of Conifers. IX. Effects of Chemical Treatments and a Wax Spray on the Outdoor Propagation of Spruce Cuttings—*N. H. Grace and J. L. Farrar* - - - 257

SECTION D.—ZOOLOGICAL SCIENCES

Storage Studies on Liquid Blood—*M. W. Thistle, N. E. Gibbons, W. H. Cook, and C. B. Stewart* - - - - - - - - 185

The Ability of Sheep's Erythrocytes to Survive Freezing—*A. H. Woodcock, M. W. Thistle, W. H. Cook, and N. E. Gibbons* 206

Canadian Wiltshire Bacon. XX. A Comparison of Certain Chemical and Physical Properties of Canadian and Danish Bacons, and Their Relation to Flavour Quality—*W. Harold White, C. A. Winkler, and W. H. Cook* - - - - - - - 213

NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

Publications and Subscriptions

The Canadian Journal of Research is issued monthly in four sections, as follows:

A. Physical Sciences
B. Chemical Sciences
C. Botanical Sciences
D. Zoological Sciences

For the present, Sections A and B are issued under a single cover, as also are Sections C and D, with separate pagination of the four sections, to permit separate binding, if desired.

Subscription rates, postage paid to any part of the world (effective 1 April, 1939), are as follows:

	Annual	*Single Copy*
A and B	$ 2.50	$ 0.50
C and D	2.50	0.50
Four sections, complete	4.00	—

The Canadian Journal of Research is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. All correspondence should be addressed:

National Research Council, Ottawa, Canada

Notice to Contributors

Fifty reprints of each paper are supplied free. Additional reprints, if required, will be supplied according to a prescribed schedule of charges.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 19, SEC. C. JULY, 1941 NUMBER 7

THE REACTION OF *FRAGARIA VIRGINIANA* TO THE VIRUS OF YELLOW-EDGE[1]

BY A. A. HILDEBRAND[2]

Abstract

When runner-grafted to domestic plants of the strawberry varieties Premier and Glen Mary (symptomless carriers of the virus of yellow-edge) clones of the common wild strawberry, *Fragaria virginiana* Duchesne, were found to vary widely in their resistance and susceptibility to the disease. Certain clones of noticeably more delicate growth type, proved to be very highly susceptible to deterioration and exhibited complete symptom-expressing propensities. Other clones, characterized by a particularly robust type of vegetative growth, although readily susceptible, showed capacity for at least partial recovery, tending to react more like the English indicator variety, Royal Sovereign. Still other clones, also of the robust type, showed resistance that was virtually complete; thus they were eliminated from plants of the "carrier" class.

Introduction

Before continuing the strawberry virus investigations carried out at St. Catharines from 1933 until 1935 (4), it became necessary to find an indicator plant to replace the English variety, Royal Sovereign, which had been used in the earlier experiments. Under Ontario conditions, plants of this variety proved so susceptible to attack by certain insect pests and fungous diseases that it was found impossible to maintain them for any considerable time at the level of health and vigour required of plants to be used in virus research. This necessitated frequent renewals of stock by importation from England and almost invariably only a few, if any, plants of each shipment survived the long period in transit. Since Harris (3) in England had demonstrated that species of *Fragaria*, namely, *F. vesca* and *F. virginiana* could be used as indicator plants, it was decided to investigate the possibility of using the same two species, both of which are indigenous to Ontario, in further work at St. Catharines. The results which have been obtained from investigations carried out during the past three years are reported in the present paper.

Experiments—1937

In May, 1937, 50 plants of *F. virginiana* and the same number of *F. vesca* were transferred to the greenhouse from their natural habitat. At the same time, Premier plants chosen from stocks obtained from two widely separated commercial sources in Ontario (hereinafter designated Premier *A* and Premier

[1] *Contribution No. 662 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.*

[2] *Assistant Plant Pathologist, Dominion Laboratory of Plant Pathology, St. Catharines, Ont.*

B), as well as plants of the variety Glen Mary, were also grown in the greenhouse. By early July of 1937, both wild and domestic plants were producing runners in profusion but those of *F. vesca* were so fine and thread-like that they could not be used for grafting and the species was discarded.

Using the method of runner inarching described by Harris in 1932 (1) and by Harris and Hildebrand in 1937 (4), runners of six, ten, and nine different

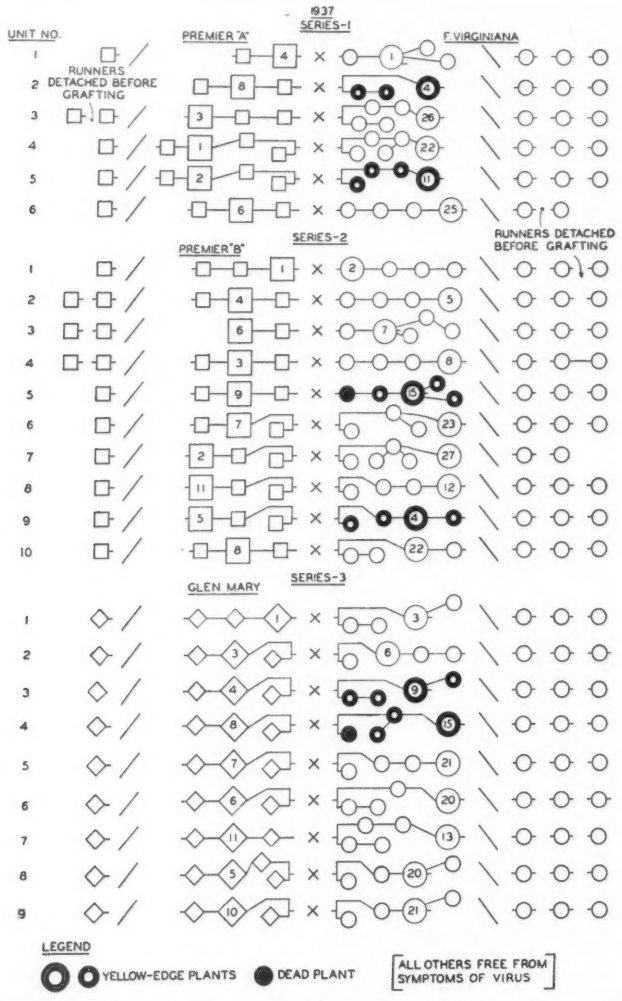


FIG. 1. *Various types of graft combinations or graft units together with results obtained in 1937 grafting experiments involving different clones of F. virginiana and domestic plants of unknown virus potentiality.*

clones of Premiers *A* and *B* and of Glen Mary, respectively, were grafted to those of *F. virginiana* plants representing 20 different clones. In some, the stolons grafted were those produced by parent plants (Fig. 1, Series 2, Unit 1). Frequently, stolons of *F. virginiana* grew to a length of several feet with "runner buds" occurring at intervals. From these it was possible to strike a number of runners which constituted a chain of plants connected in series by a common stolon. The continuation of the latter from the terminal plant of the series was used for grafting (Fig. 1, Series 1, Unit 6). In a graft unit of the latter type it was thought that in the event of transmission of virus from the Premier or Glen Mary component it might be possible to trace the rate of spread of the disease by noting the time interval elapsing between symptom expression in successive "links" in the chain of *F. virginiana* plants organically united in series.

At the commencement of the experiment all plants involved were in an apparently healthy condition. To avoid complications that might arise from a root rot factor, all runner progeny were grown in autoclaved soil. The 25 graft units completed in July, 1937, were kept under constant observation in the greenhouse until June, 1938. The arrangement and number of plants involved in the 1937 series, together with the end results of the experiment are shown graphically in Fig. 1.

Results

The *F. virginiana* components of six graft units developed symptoms which were indistinguishable from those of yellow-edge on Royal Sovereign. Furthermore, the virus was transmitted to only four of the 20 different clones of *F. virginiana*, namely, 4, 9, 11, and 15 (Fig. 1). Of the four apparently susceptible clones, 4 and 11 exhibited a particularly robust vegetative growth and without close examination could scarcely be distinguished from plants of cultivated varieties. Clones 9 and 15, on the other hand, were characterized by a noticeably more fragile type of growth, their leaves, petioles, and stolons being much less robust than those of the other clones—including 4 and 11. These differences gave evidence of the wide variations that must exist within the species as far as vegetative characters are concerned.

The reaction of plants of the wild species to yellow-edge seemed correlated in part, at least, with vigour of vegetative growth. For example, the disease was much more severe in plants of Clones 9 and 15 than in those of 4 and 11. Moreover, plants of Clones 9 and 15, once infected, remained permanently in the dwarfed, "flattened" condition, whereas those of Clones 4 and 11 showed capacity for at least partial recovery from the disease, especially during the period of renewed growth activity in the spring of 1938. Thus, plants of Clones 4 and 11 tended to react more like those of the English indicator variety, Royal Sovereign.

In Series 3, Graft unit 4 (Glen Mary 8 $\times$*F. virginiana* 15), yellow-edge symptoms became apparent on the *F. virginiana* plant closest to the graft union about 22 days after the date of grafting. In the corresponding *F. vir-*

giniana plant, Series 2, Graft unit 5 (Premier B 9 × *F. virginiana* 15), the symptoms became distinguishable about 30 days after the graft had been made. In no other case did symptoms show up in *F. virginiana* plants grafted directly to either Premier or Glen Mary plants in less than 60 days after grafting. This circumstance and the fact that before the expiration of the experiment two plants of Clone 15 died (Fig. 1, Series 2, Graft unit 5; Series 3, Graft unit 4), made it appear that this clone was even more susceptible than Clone 9 and much more so than Clones 4 and 11.

All progeny of the apparently susceptible Clones 4, 9, 11, and 15 that had been detached previous to grafting, remained free from symptoms of yellow-edge, as did all other plants, wild and domestic, involved in the experiment. Non-appearance of virus symptoms could not be attributed in a single instance to faulty technique since in the 25 graft units organic union had taken place at the point of grafting.

Discussion

Since previous work at St. Catharines (4) had demonstrated that Premier and Glen Mary plants both possess the symptomless-carrier capacity for yellow-edge and since Harris (3) had shown also that *F. virginiana* "proved to be highly susceptible to deterioration and exhibited all symptoms" (of yellow-edge), no especial interest attached to the fact of merely having confirmed these findings. It did seem of particular interest, however, that as the results suggested, within the species there might exist clones or strains apparently differing so widely in their resistance and susceptibility to the disease. It was realized, of course, that non-appearance of yellow-edge symptoms in the *F. virginiana* components of 19 of the 25 graft units might have been owing to the fact that certain of the domestic plants were free from infection, as had been found in previous work; nor was it impossible that certain of the wild plants might have possessed the symptomless-carrier capacity to as high degree as the Premier and Glen Mary plants to which they were grafted; in that case, though infected, they would not show symptoms. To try to arrive at the true explanation of the observed phenomena, further work was carried out in 1938.

Experiments—1938

Early in July, 1938, there was completed a fourth series of grafts (Fig. 2) in which progeny of plants of the commercial varieties, Glen Mary and Premier, that in 1937 *had passed on* the virus, were grafted to *F. virginiana* plants of the clones that had *failed to take* the disease. Reciprocally, Glen Mary and Premier plants that apparently *had not passed on* the virus were grafted to healthy *F. virginiana* plants of the clones that *had taken* it from other plants of these two varieties. Altogether, as reference to Fig. 2 will show, Series 4 comprised 12 such graft units.

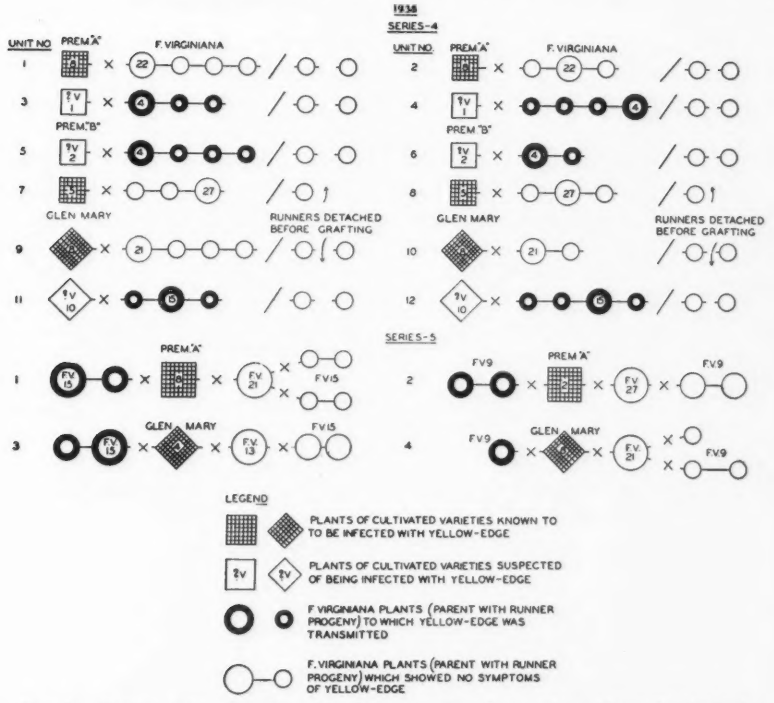


FIG. 2. *Various types of graft combinations or graft units, together with results obtained in 1938 grafting experiments involving presumably resistant and susceptible clones of F. virginiana and domestic plants of known and suspected virus potentiality.*

Although the number of domestic plants of known history, with runners suitable for grafting, was limited, sufficient were still available to complete the four additional graft units which comprised Series 5. This series was designed primarily to find out whether certain clones of the wild plants which up to that time had failed to show symptoms of the disease, were really resistant to it, or like certain clones of the domestic varieties, were susceptible but possessed the symptomless-carrier capacity. Details of the arrangement of plants and scheme of grafting are shown in Fig. 2, Series 5, Graft units 1 to 4. A domestic plant, proved in 1937 to be infected with yellow-edge, was grafted by one runner to a wild plant known to be susceptible and by another to a presumably resistant wild plant. The "resistant" wild plant in turn was grafted to one or (if possible) two of the susceptible wild plants. Thus was constituted a "chain" of plants the respective "links" being as follows: susceptible wild plant, domestic plant of known virus content, resistant wild plant, and again the susceptible wild plant. The results obtained in the 1938 grafting are shown graphically in Fig. 2.

Results

Series 4

F. virginiana plants of Clones 21, 22, and 27, when grafted to Premier and Glen Mary plants proved by the work of 1937 to be infected with yellow-edge, failed to show any symptoms of the disease. It remained to be seen whether or not these *F. virginiana* clones had become infected. If so, it could then be assumed that they must possess the symptomless-carrier capacity to as high degree as the domestic plants to which they were grafted. If not, they must be completely resistant. In each case where *F. virginiana* plants of presumably susceptible Clones 4 and 15, were grafted to Premier and Glen Mary plants of as yet unknown virus potentiality, they developed characteristic symptoms of the disease, thus confirming earlier indications that they were really susceptible to infection by the virus and by the same token proving that Premier *A* 1, Premier *B* 2 and Glen Mary 10 were infected with the disease. As indicated in Fig. 2, no symptoms of yellow-edge were noted on runner plants of any of the *F. virginiana* clones, detached before the grafts were made. The above results seemed to furnish clear evidence that clones of the wild plants differed markedly in resistance and susceptibility to the disease.

Series 5

In all four cases *F. virginiana* plants of susceptible Clones 15 and 9, grafted directly to the virus infected domestic plants, developed characteristic symptoms of the disease. In contrast, however, *F. virginiana* plants of the "resistant" Clones 13, 21, and 27, also grafted directly to the virus infected domestic plants, showed no evidence of the disease throughout the period of observation which lasted from the time the grafts were made in July, 1938, until June of the following year. That plants of Clones 13, 21, and 27 must be regarded as possessing resistance that is virtually complete, is proved by the fact that plants of the highly susceptible Clones 9 and 15, which formed the final link in the chain of grafted plants, showed no evidence of the disease. Examination showed that organic union had taken place at each point of grafting so that failure of transmission could not be attributed in a single instance to faulty technique. The same was true for all graft units of Series 4.

Experiments—1939

As in previous years progeny of the various clones of wild and domestic plants were carried through the winter of 1938–39. In the spring and early summer of 1939, the wild plants produced an abundance of runners suitable for grafting. In marked contrast, however, the domestic plants produced so few runners that it was impossible to build up a population of these plants and further work could not be carried out as planned. If additional grafting were to be done, the only alternative was to resort again to domestic plants of unknown virus potentiality. Late in June, Glen Mary and Premier plants, the latter, as in 1937, obtained originally from two widely separated sources

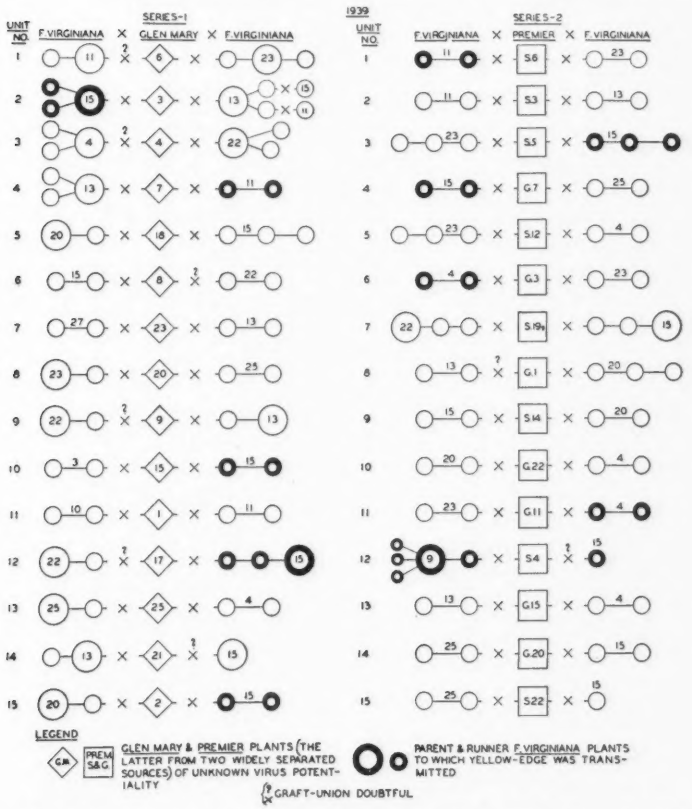


FIG. 3. *Various types of graft combinations or graft units, together with results obtained in 1939 grafting experiments involving presumably resistant and susceptible clones of F. virginiana and domestic plants of unknown virus potentiality.*

in Ontario, were brought into the greenhouse from outdoor plots. As runners from these plants became available, two series, each comprising 15 graft units, were completed. In Series 1, a runner from each of two different clones of *F. virginiana* was grafted to each of a pair of runners produced by a Glen Mary plant. Series 2 was the same except that Premier plants were used, the designations *S* and *G* indicating their different sources. As reference to Fig. 3 will show, both of the *F. virginiana* components in certain of the graft units (Series 1, Unit 8) represent resistant clones. In other graft units (Series 2, Unit 12) both of the *F. virginiana* components represent susceptible clones. In still others (Series 1, Unit 15), one *F. virginiana* component represents a resistant clone, the other a susceptible one. It will also be noted that in a total of 60 grafts, the success of the graft in eight was doubtful. These eight possible failures are probably owing to the fact that because of the change in

plans necessitated by the non-production of runners by the domestic plants used in 1937 and 1938, a considerable period elapsed between the time of runner production by the wild plants and the formation of runners by the new series of domestic plants. This meant that in a number of grafts the runners used, particularly those of the wild plants, had passed the stage of succulence most conducive to success in grafting. Furthermore, some of the grafts could not be completed until about the middle of August. Previous experience has shown that much greater success is obtained when grafts are made earlier in the growing season. The results obtained in the 1939 experiments are shown graphically in Fig. 3.

As reference to Fig. 3 will show, in the 43 grafts in which representatives of presumably resistant clones were grafted to the domestic plants of the two varieties, symptoms of yellow-edge did not appear on any of the wild-plant components, thus confirming previous evidence as to their resistance to the disease. In 12 of the 27 grafts of representatives of susceptible clones to the domestic plants, characteristic symptoms of yellow-edge resulted. In three of the remaining 15, involving presumably susceptible clones (Series 1, Units 1, 3, and 14), failure of transmission might reasonably be attributed to failure of organic union at the point of grafting. In the non-appearance of yellow-edge in the remaining 12 grafts of *F. virginiana* plants from susceptible clones and Premier and Glen Mary plants, it is probable that the latter were not infected with the virus. In previous work (4) it was found that certain Glen Mary and Premier plants apparently were not infected since they did not transmit the disease to plants of the highly susceptibe variety, Royal Sovereign.

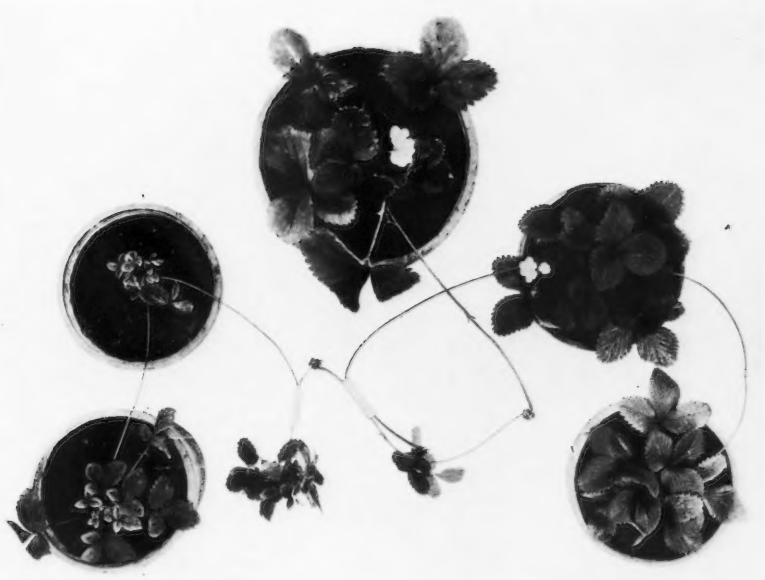
Attention should be directed to Unit 2 of Series 1. Glen Mary 3 has transmitted the virus to susceptible *F. virginiana* 15. However, *F. virginiana* 13 was evidently completely resistant to the disease, otherwise the virus would have been transmitted through it to *F. virginiana* 11 and 15, both of which are susceptible. This unit offers further confirmation to the conclusion drawn from the results of Series 5, 1938, that certain strains of *F. virginiana* are virtually completely resistant to the disease.

As typical of a graft unit showing very clearly two clones of the wild plant differing markedly in their resistance and susceptibility to the disease, Unit 6, Series 2 (Fig. 3) was photographed (Plate I) in October, 1939, approximately three months after the grafts had been made.

Discussion

In England where virus diseases are "a serious and widespread contributory cause of varietal deterioration" (3, p. 201) intensive research has been carried out almost continuously since 1932 when Harris (2) identified yellow-edge on the variety Royal Sovereign. The study of yellow-edge, at first centring about the above named variety, has been extended to include other commercial varieties and species of *Fragaria*. The results as summarized recently by

PLATE I



Graft unit 6, Series 2, 1939, typical of many others showing resistant (right) and susceptible (left) clones of F. virginiana grafted to yellow-edge—infected Premier plant (upper centre) of the "carrier" type. Unit photographed approximately three months after grafts were made.

Harris and King (5) are in part as follows— "*Fragaria chiloensis* reacted as a symptomless carrier of the disease with high resistance to deterioration and at the other end of the scale *F. virginiana* (cultivated in this country as "Little Scarlet") and the native woodland species, *F. vesca*, were found to combine a high degree of symptom expression with extreme susceptibility to deterioration. Preliminary trials have further indicated that the existing commercial varieties form a series between the extremes represented by their parent species (*F. chiloensis* and *F. virginiana*), some varieties, e.g., Lefebvre and Huxley's Giant approximating in reaction to the former, and others, e.g., Royal Sovereign and Sir Joseph Paxton, to the latter species."

From the above it would appear that the English workers have not yet encountered plants among the wild species or domestic varieties that show as high resistance to yellow-edge as do those of certain clones of *F. virginiana* indigenous to Ontario. It is intimated by Harris and King (5) that "samples of *F. indica* and *F. virginiana* (from North American sources)" are to be used in further tests as to their efficiency as indicator plants. It will be interesting to await the results of these tests, especially as regards *F. virginiana*, to see how they compare with those reported in the present paper.

Of interest in the present studies was the observance, first, of such wide variations in inherent vigour of growth among clones of the wild species and then, the discovery of the apparent correlation between vigour of vegetative growth and resistance and susceptibility to the disease. From investigations along other lines which have been carried out at St. Catharines during recent years, it was learned that *F. virginiana* plants are in general much more resistant to root rot than those of any of the cultivated varieties tested. These findings together with those reported in the present paper suggest implications of practical importance. In breeding new varieties of strawberries involving *F. virginiana* as one of the parents, it would seem highly desirable to employ clones of the latter, which, in addition to outstanding vigour of growth, possess the important character of resistance to root rot and to the virus of yellow-edge.

References

1. HARRIS, R. V. J. Pomology Hort. Sci. 10 : 35-41. 1932.
2. HARRIS, R. V. J. Pomology Hort. Sci. 11 : 56-76. 1933.
3. HARRIS, R. V. Ann. Rept. East Malling Research Sta. for 1936 : 201-211. 1937.
4. HARRIS, R. V. and HILDEBRAND, A. A. Can. J. Research, C, 15 : 252-280. 1937.
5. HARRIS, R. V. and KING, M. E. Ann. Rept. East Malling Research Sta. for 1939 : 66-68. 1940.

VARIETAL DIFFERENCES IN BARLEYS AND MALTS

XI. SIMULTANEOUS RELATIONS BETWEEN MALT EXTRACT AND TWO OR MORE BARLEY PROPERTIES[1]

By H. R. Sallans[2], W. O. S. Meredith[3], and J. A. Anderson[4]

Abstract

Inter- and intravarietal relations between malt extract and barley properties (extract, starch, total nitrogen, 1000-kernel weight, salt-soluble nitrogen, saccharifying activity, cellulose-lignin residue, and steeping time) have been investigated by developing prediction equations for malt extract.

The most useful single factors for intervarietal prediction are barley extract, starch, and cellulose-lignin residue, in the order given. The inclusion of salt-soluble nitrogen and steeping time, as additional independent variables, with barley extract or starch, results in a significant improvement in the level of prediction. The most accurate equation was: *malt extract* = $1.1 + 0.93$ *barley extract* $+ 7.44$ *salt-soluble nitrogen* $- 0.035$ *steeping time*. This equation serves to indicate the relative extract yield of varieties grown at the same station, the standard error being $\pm 0.8\%$. The constant, 1.1, varies from station to station; hence, although this average value gives relative extract yields, the constant must be evaluated for specific environments if absolute extract yields are required.

The most useful single factors for intravarietal prediction are barley extract, starch, and total nitrogen. Statistical analysis showed that only barley extract and total nitrogen could be effectively combined for prediction purposes. The equation is: *malt extract* = $A + 0.58$ *barley extract* $- 2.4$ *total nitrogen*, standard error $\pm 0.6\%$. The factor A is dependent on variety but the data suggest that for Canadian malting varieties this constant has a value of about 35.7.

Attempts to develop a generalized equation applicable to all samples, irrespective of varieties and the environment in which they were produced, proved unsuccessful. This is apparently due to significant differences between the inter- and intravarietal partial regression coefficients for the properties studied.

In previous papers in this series, the relations between malt extract and each of a number of barley properties were examined and the possibilities of predicting the former from data on one of the latter were discussed. In the present paper, the treatment of the data is expanded to include studies of the simultaneous relations between malt extract and two or more barley properties and of the possibilities of developing prediction equations containing more than one independent variable.

Data on 12 varieties of barley grown at 12 Canadian experimental stations in 1937 (1–5, 10–13) were used in the present study. In addition, the more interesting relations were re-examined in the light of an additional set of data (not yet published) for 24 varieties grown at six Canadian experimental stations in 1938.

[1] *Manuscript received February 11, 1941.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Published as Paper No. 183 of the Associate Committee on Grain Research and as N.R.C. No. 996.

[2] *Formerly Biochemist, National Research Laboratories, Ottawa; now Biochemist, Oil Seeds Laboratory, University of Saskatchewan, Saskatoon, Sask.*

[3] *Senior Research Assistant, Associate Committee on Grain Research, Department of Plant Science, University of Manitoba, Winnipeg, Man.*

[4] *Formerly Biochemist, National Research Laboratories, Ottawa; now Chief Chemist, Grain Research Laboratory, Board of Grain Commissioners, Winnipeg.*

In considering the subject of prediction, certain conceptions must be kept clearly in mind. It is apparent that differences, with respect to any barley or malt property, that exist between samples of different varieties grown at the same station, result from genetic or hereditary dissimilarities whereas differences that exist between samples of the same variety (from different stations) result from dissimilarities in the environments under which the samples were grown. Moreover, it follows that such intervarietal associations as occur between any two properties are governed by hereditary factors and that intravarietal associations are governed by environmental factors. In general, when an association between two properties results from a fundamental and close relationship between these properties, this association will exist both within and between varieties and will be invariable irrespective of the individual varieties or environments represented by the samples from which the association is deduced. On the other hand, there exist certain associations between barley and malt properties that do not represent close and fundamental relations but appear to reflect complex interrelations between a number of properties. Associations of the latter type may exist within varieties but not between varieties, or vice versa, or may exist both within and between varieties, but in such a way that the inter- and intravarietal relations obviously differ in character. All these types of associations have been found in the present investigation and the necessity for making separate examinations of inter- and intravarietal correlations has thus been amply established. The work thus leads to the development of certain equations for the prediction of malt extract that can be applied only to samples of different varieties grown under the same environmental conditions, of other equations that can be applied only to samples of one variety, or closely related group of varieties (cf. 6–9), and finally to the study of general equations which are applicable to all barley samples irrespective of variety or origin.

Intervarietal Prediction Equations

In developing an equation for the prediction of malt extract based on two barley properties, it is logical to select, as one of the latter, a property that is closely associated with malt extract. The degrees of association between malt extract and the more important barley properties studied in the 1937 investigation (in which 12 varieties were grown at each of 12 stations) are shown by the simple intervarietal correlation coefficients given in the first column of data in Table I. An examination of these coefficients shows that the three barley properties that show the most promise for prediction purposes are barley extract, cellulose-lignin residue, and starch.

If barley extract is used as one property, the second property that can best be combined with barley extract, can be selected by considering partial correlation coefficients, independent of barley extract, between malt extract and each remaining barley property. These partial correlation coefficients are given in the second column of data in Table I. The remaining two columns

of data give the corresponding partial correlation coefficients, independent of starch and independent of cellulose-lignin.

TABLE I

INTERVARIETAL CORRELATION COEFFICIENTS BETWEEN MALT EXTRACT AND CERTAIN BARLEY PROPERTIES

Barley property	Correlation coefficient			
	Simple	Partial, independent of:		
		Barley extract	Starch	Cellulose-lignin residue
Total nitrogen	.350	−.039	−.084	−.195
Alcohol-soluble nitrogen	−.200	−.235	−.286	−.417
Insoluble nitrogen	−.728**	−.577	−.545	−.366
Acid-resistant nitrogen	−.816**	−.450	−.510	−.298
Cellulose-lignin residue	−.912**	−.267	−.624*	—
Starch	.854**	−.536	—	−.105
Barley extract	.914**	—	.776**	.291
Saccharifying activity	−.001	.316	.695**	.674*
Salt-soluble nitrogen	.452	.876**	.839**	.640*
Steeping time	−.589*	−.791**	−.859**	−.814**
1000-kernel weight	.439	−.464	−.398	−.374

NOTE: *In this and later tables* (*) *indicates that a 5% level of significance and* (**) *that a 1% level of significance is attained.*

Only two of the partial correlation coefficients between malt extract and barley properties, independent of barley extract, are significant. These are the coefficients for salt-soluble nitrogen and for steeping time. Accordingly, it would appear that the prediction of malt extract from barley extract can be improved by inclusion in the prediction equation of either salt-soluble nitrogen or steeping time. Since the remaining partial correlation coefficients in the second column of data are not significant, none of the other barley properties can be combined advantageously with barley extract for prediction purposes.

Inspection of the last two columns of data in Table I will show that for predicting malt extract, both salt-soluble nitrogen and steeping time also lend themselves best to combination with either starch or cellulose-lignin residue. Significant but lower partial correlation coefficients, independent of starch, are also given by malt extract and each of the following three barley properties: —cellulose-lignin residue, barley extract, and saccharifying activity. However, although the partial correlation coefficient between malt extract and cellulose-lignin residue, independent of starch, is significant, that between malt extract and starch, independent of cellulose-lignin residue is not significant. It would thus appear that the negative relation between malt extract and cellulose-lignin residue (i.e., the positive relation between malt extract and the acid-soluble portion of barley) is closer than the relation between

malt extract and starch. This can readily be conceived since it is apparent that malt extract includes, in addition to the starch, certain other soluble and readily hydrolysable components of the barley which are removed by treatment with boiling acid. Thus, for purposes of predicting malt extract, there is no advantage in combining starch with cellulose-lignin residue as is shown by the fact that the partial correlation coefficient for malt extract and this residue, independent of starch, is not significant. Exactly the same situation exists with respect to the relations between malt extract, barley extract, and starch; no significant improvement in the prediction of malt extract from barley extract can be obtained by including starch as a second independent variable.

The associations between malt extract, barley starch, and the saccharifying activity of the barley are somewhat more complex. Whereas the simple correlation between extract and saccharifying activity is not significant, the partial correlation between these two properties, independent of starch, is significant. It thus appears that there is a direct association between extract and saccharifying activity, but this is masked in the simple correlation by the associations that exist between each of these properties and starch content. The statistics suggest that in developing an equation for the prediction of malt extract, it might be worth while to consider the simultaneous relation between extract, on the one hand, and starch and saccharifying activity, on the other.

Having selected those pairs of properties that seem most promising for prediction purposes, the multiple correlation coefficients for malt extract and each pair can now be examined. To these may be added certain multiple correlations involving three rather than two independent variables. The various correlation coefficients are listed in the first column of data in Table II. The next column shows the results of analyses of variance undertaken to determine whether the addition of each independent variable raises the correlation coefficient significantly.

The study of data on samples of 12 varieties grown at each of 12 stations in 1937 was supplemented with a further study of 24 varieties grown at 6 stations in 1938. In the latter study, only those barley properties and associations, that had appeared most useful in previous work, were re-examined. The resulting correlation coefficients, together with information on the significance of added variables, are given in the last two columns of Table II.

The 1937 data for variety means show that several pairs of barley properties are correlated with malt extract to about the same degree. These pairs include: *(a)* barley extract and salt-soluble nitrogen or steeping time and *(b)* starch and each of the two last named properties. In each case the multiple correlation coefficient is significantly higher than the corresponding simple coefficient. When salt-soluble nitrogen and steeping time are combined with barley extract, giving three independent variables, the slight improvement in the multiple correlation is not significant whereas, if starch is substituted for barley extract, the improvement in the multiple correlation coefficient attains the 5% level of significance.

TABLE II

INTERVARIETAL CORRELATION COEFFICIENTS BETWEEN MALT EXTRACT AND CERTAIN BARLEY PROPERTIES

Barley property	1937 data[1]		1938 data[2]	
	Correlation coefficient	Significance of added variable	Correlation coefficient	Significance of added variable
Barley extract	.914**	—	.854**	—
Barley extract × salt-soluble nitrogen	.980	**	.919**	**
Barley extract × steeping time	.969**	**	.906**	**
Barley extract × salt-soluble nitrogen × steeping time	.984**	0	.944**	**
Starch	.854**	—	.785**	—
Starch × saccharifying activity	.927**	*	.826**	*
Starch × salt-soluble nitrogen	.959**	**	.939**	**
Starch × steeping time	.964**	**	.892**	**
Starch × salt-soluble nitrogen × steeping time	.976**	*	.972**	**
Acid-resistant residue	−.912**	—	—	—
Acid-resistant residue × salt-soluble nitrogen	.939**	0	—	—
Acid-resistant residue × steeping time	.971**	**	—	—
Acid-resistant residue × salt-soluble nitrogen × steeping time	.972**	*	—	—

[1] 12 varieties grown at 12 stations in 1937.
[2] 24 varieties grown at 6 stations in 1938.

The multiple correlation coefficients for malt extract, starch, and saccharifying activity and for malt extract, cellulose-lignin residue, and other properties are somewhat lower and suggest that these various combinations are less useful for prediction purposes. It was for this reason that cellulose-lignin residues were not studied again in 1938.

In 1938, when 24 varieties comprising a wider range were used, the correlation coefficients proved to be somewhat lower, as might be anticipated. Among the correlations involving two independent variables, that for starch and salt-soluble nitrogen gave the highest coefficient whereas, for the 1937 data, the highest coefficient was given by barley extract and salt-soluble nitrogen. In 1938, as in 1937, the correlation between malt extract, starch, salt-soluble nitrogen, and steeping time, proved to be significantly higher than those correlations in which one of the last two variables was omitted. A similar situation existed in 1938 with respect to the corresponding correlations involving barley extract, although no improvement had been obtained by using a third independent variable in this series during the previous year.

As a result of these preliminary studies of data for two years, it appeared worth while to undertake a more exhaustive examination of the relations between malt extract and each of the following sets of barley properties:—

extract and salt-soluble nitrogen; extract and steeping time; extract, salt-soluble nitrogen, and steeping time; and the three corresponding sets of properties in which starch was substituted for barley extract.

Each of these relations between malt extract and a set of barley properties represents the equation of a surface. When two barley properties are involved, the equation takes the form:—

$$E = A + b_1P_1 + b_2P_2$$

When three barley properties are involved it becomes:—

$$E = A + b_1P_1 + b_2P_2 + b_3P_3 .$$

In each of these equations E represents the dependent variable, malt extract; P values represent the various barley properties; and A and b values are constants, the former representing the E intercept and the latter the partial regression coefficients of malt extract on each barley property. For any given set of appropriate data the partial regression coefficients (b values) and the remaining constant A can be calculated by the usual statistical methods.

We may consider, as an example, the relation between malt extract, barley extract, salt-soluble nitrogen, and steeping time derived from the 1937 data. Since these data represent a study of 12 varieties grown at each of 12 stations, 13 intervarietal prediction equations for malt extract can be computed, namely, one equation for each of the 12 stations and an average equation for all stations combined. These equations were calculated and it was found that the partial regression coefficients (b values) and the values of the constant A differed slightly for each equation. It was therefore necessary to determine whether these differences were sufficiently small to be accounted for by the experimental errors of the investigation, or whether they were large enough to indicate that the relation is not homogeneous and differs from station to station.

This examination was made by analysing the residual variance into three portions:—(i) differences among station regression coefficients, representing that part of the residual variance due to differences in the slopes of the equation surfaces (b values) for each of the 12 stations; (ii) deviations of centroids from the average regression, representing that part of the residual variance due to differences in the positions of the equation surfaces for each of the 12 stations; (iii) deviations of the observations from each of the 12 individual regression functions, a remainder that may be used as an estimate of experimental error. The results of this analysis of residual variance are given in the first two columns of data in Table III. The statistics show conclusively that the centroids (i.e., the positions of the regression surface for each station) differ significantly, but that such differences as exist between the b values (i.e., the slopes of the partial regression surfaces) may well be due to experimental errors.

The second two columns of data in Table III show the results of similar analyses of residual variance made for the 1938 data. Exactly the same

TABLE III

ANALYSIS OF RESIDUAL VARIANCE FOR THE INTERVARIETAL RELATION BETWEEN MALT EXTRACT, BARLEY EXTRACT, SALT-SOLUBLE NITROGEN, AND STEEPING TIME

Variance due to:	1937 data		1938 data		Combined data	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Differences among station regression coefficients	33	0.542	15	0.785	51	0.748
Deviations of centroids from average station regression	11	2.866**	5	9.595**	17	5.038**
Deviations from individual station regressions	96	0.670	120	0.558	216	0.608

situation exists; the b values do not differ significantly but there are significant differences in the centroids from station to station.

The b values for the two years were compared by subjecting the combined data to an analysis of residual variance. The results of this analysis appear in the last two columns of Table III and show that the regressions are homogeneous. A comparison of the residual variance for differences among regression coefficients in each of the two years gave an F value of 1.45 as compared with 2.02 for the 5% level of significance. Hence no significant differences between the b values exist between years.

In terms of the prediction equation,

$$E = A + b_1P_1 + b_2P_2 + b_3P_3 ,$$

this means that whereas the constants b_1, b_2, and b_3 can be assumed to have the same values under all environments studied, the constant A should be given a different value for each environment. In other words, a general equation can be derived in which b_1, b_2, and b_3 are given definite values but A is not evaluated. This equation will serve for predicting the differences between any pair of varieties grown at the same station, or at the same set of stations, but it will not serve for the prediction of the actual yield of malt extract given by any variety grown at a certain station, unless the appropriate A value for that particular station is known.

For certain purposes this limitation is not important. Thus a plant breeder will generally wish to compare a new variety with a standard variety grown under the same conditions. Under these circumstances, it will be sufficient to know whether the predicted malt extract yield of the new variety is higher or lower than that of the standard variety and to what extent. Knowledge of the actual levels of malt extract predicted for the two varieties is of little importance. If it is known that a new variety will probably produce an average of 2% more extract than the standard variety, when grown at a certain set of stations, it is of little significance whether the actual values for the varieties turn out to be 74 and 72%, or 72 and 70%.

TABLE IV

ANALYSIS OF RESIDUAL VARIANCE FOR INTERVARIETAL PREDICTION OF MALT EXTRACT FROM EQUATIONS INVOLVING VARIOUS COMBINATIONS OF TWO OR THREE BARLEY PROPERTIES

Data	Variance due to:	Degrees of freedom	Mean square				Degrees of freedom	Mean square	
			Barley extract and:		Starch and:			Salt-soluble nitrogen, steeping time, and:	
			Salt-soluble nitrogen	Steeping time	Salt-soluble nitrogen	Steeping time		Barley extract	Starch
1937	Differences among station regression coefficients	22	0.644	0.832	0.521	1.126	33	0.542	0.572
	Deviations of centroids from average station regression	11	5.584**	3.281**	6.207**	4.099**	11	2.866**	5.144**
	Deviations from individual station regressions	108	0.691	0.623	1.026	0.795	96	0.670	0.723
1938	Differences among station regression coefficients	10	1.033	1.604*	2.374**	1.837	15	.785	1.693**
	Deviations of centroids from average station regression	5	8.659**	4.291**	25.419**	12.050**	5	9.595**	25.162**
	Deviations from individual station regressions	126	0.673	0.741	0.830	1.271	120	0.558	0.749

The various combinations of barley extract, starch, salt-soluble nitrogen, and steeping time listed in Table II were examined in a similar manner and the results of the analysis of residual variance are collected in Table IV. From the data it is evident that in all cases there are significant deviations of the centroids from the average regressions and consequently the factors A cannot be evaluated for the preparation of generalized equations applicable to all stations. However, for comparing varieties grown at the same station, or group of stations, in the same year, an average value of A may be used effectively. The data also show that the partial regression coefficients, or b values, are homogeneous within years for the relations between malt extract and the following barley properties: *(a)* barley extract and salt-soluble nitrogen; *(b)* starch, steeping time, and barley extract; *(c)* salt-soluble nitrogen and steeping time. Furthermore, it can be shown that the b values for these relations do not differ significantly between years. Thus these relations may be used in the preparation of intervarietal prediction equations valid for both the 1937 and 1938 data.

Intervarietal prediction equations are listed below. With the exception of the sixth, these are based on two years' data representing 288 samples; the final equation involves cellulose-lignin residue that was derived from the 1937 data and represents 144 samples. The standard error of prediction for a single sample is given in parentheses after each equation. In general, it will not prove satisfactory to test new varieties by growing them at one station only, owing to the fact that they do not always fall in exactly the same order

with respect to malt extract when grown at different stations. To overcome this difficulty the authors recommend growing the varieties at a minimum of four stations to obtain reasonably accurate estimates of potential yields of malt extract. If this is done the standard error of estimate of the variety means will become approximately one-half the value indicated after each equation.

INTERVARIETAL PREDICTION EQUATIONS

$$E = 1.0 + 0.96\,B \qquad (\pm 0.98)$$
$$E = -4.0 + 0.94\,B + 10.4\,S.N \qquad (\pm 0.86)$$
$$E = 1.1 + 0.93\,B + 7.44\,S.N - 0.035\,S.T \qquad (\pm 0.80)$$
$$E = 25.5 + 0.89\,S \qquad (\pm 1.21)$$
$$E = 29.7 + 0.90\,S - 0.067\,S.T \qquad (\pm 1.07)$$
$$E = 49.9 - 2.02\,C \qquad (\pm 1.25)$$

In the above equations, E = malt extract, B = barley extract, $S.N$ = salt-soluble nitrogen, $S.T$ = steeping time, S = starch, and C = cellulose-lignin residue.

Intravarietal Prediction Equations

The simple intravarietal correlation coefficients for malt extract and various barley properties are given in the first column of data in Table V. Examination of these will show that malt extract is most highly correlated with barley extract, starch content, total nitrogen, and alcohol-soluble nitrogen. In presenting the partial correlation coefficients given in the remaining columns of the table, those independent of alcohol-soluble nitrogen have been left out since they are essentially similar to corresponding coefficients independent of total nitrogen.

Superficially it might appear that starch could be combined with barley extract but since the correlation between malt extract and starch, independent

TABLE V

INTRAVARIETAL CORRELATION COEFFICIENTS BETWEEN MALT EXTRACT AND CERTAIN BARLEY PROPERTIES

Barley property	Correlation coefficient			
	Simple	Partial, independent of:		
		Barley extract	Starch	Total nitrogen
Total nitrogen	−.957**	−.755**	−.453	—
Alcohol-soluble nitrogen	−.958**	−.835**	−.571	−.223
Insoluble nitrogen	−.883**	−.416	−.199	.022
Acid-resistant nitrogen	−.940**	−.711*	−.103	.119
Cellulose-lignin residue	−.329	.380	.188	−.569
Starch	.967**	.501	—	.625*
Barley extract	.973**	—	.622*	.854**
Saccharifying activity	.861**	−.682*	−.331	.037
Salt-soluble nitrogen	.767**	−.304	.046	.306
Steeping time	.722**	−.524	−.289	.503
1000-kernel weight	.600*	−.412	−.349	.416

of barley extract, is not significant, it is apparent that the combination of starch with barley extract will not prove advantageous.

A similar situation exists with respect to the combination of total nitrogen with starch. The partial correlation independent of total nitrogen is significant but that independent of starch is not. Consequently the combination will not prove useful. The only other partial correlation, independent of total nitrogen, which is significant, is that between malt extract and barley extract. Accordingly, as previously noted, the prediction of malt extract from barley extract and total nitrogen will prove to be significantly better than the prediction from either factor alone.

TABLE VI

INTRAVARIETAL CORRELATION COEFFICIENTS BETWEEN MALT EXTRACT AND CERTAIN BARLEY PROPERTIES

Barley property	Correlation coefficient	Significance of added variable
Barley extract	.973**	—
Barley extract × total nitrogen	.989**	**
Barley extract × acid-resistant nitrogen	.982**	0
Barley extract × saccharifying activity	.986**	*
Starch	.967**	—
Total nitrogen	−.957**	—
Total nitrogen × 1000-kernel weight	.964**	0

The multiple correlation coefficients for malt extract and those combinations of factors discussed above, are given in Table VI. The correlation coefficient for malt extract, total nitrogen, and 1000-kernel weight is also included since it has previously been examined by Bishop (6–8). The last column in Table VI shows the results of analyses made to determine whether the addition of the second independent variable raised the correlation coefficient significantly.

Inspection of the table will show that a significant increase in the correlation coefficient for malt extract and barley extract occurs when total nitrogen or saccharifying activity is added as a second independent variable. The influence of saccharifying activity on this relation is apparently due to the

TABLE VII

ANALYSIS OF RESIDUAL VARIANCE BY VARIETIES FOR PREDICTION OF MALT EXTRACT FROM THE MULTIPLE FACTORS TOTAL NITROGEN AND BARLEY EXTRACT

Variance due to:	Degrees of freedom	Mean square
Differences among varietal regression coefficients	22	0.46437
Deviations of centroids from average varietal regression	11	9.02368**
Deviations from individual varietal regressions	108	0.33224

close intravarietal association between saccharifying activity and total nitrogen (3, 9). Consequently, only the relation involving total nitrogen is considered in the following discussion. An analysis of residual variance, given in Table VII, shows that for this relation there are significant differences in the centroids for different varieties but not in the regression coefficients.

The prediction equation is as follows:—

$$\text{Malt extract} = A + 0.58 \text{ barley extract} - 2.4 \text{ total nitrogen } (\pm 0.6).$$

The constant A has the following values for the different varieties studied:—

O.A.C. 21	35.7	Nobarb	33.8	Charlottetown	36.0
Mensury	35.8	Regal	33.8	Hannchen	36.1
Olli	36.6	Wisconsin	33.4	Victory	35.4
Peatland	35.1	Velvet	34.8		
Pontiac	34.8				

It will be observed that these values range from 36.6 for Olli to 33.4 for Wisconsin, so that if an attempt were made to use the same value for each variety a considerable error would be introduced. However, certain pairs of varieties have closely similar values. These include:— the two closely related rough-awned six-rowed varieties, O.A.C. 21 and Mensury; the two smooth-awned six-rowed varieties, Nobarb and Regal; and the two-rowed varieties, Charlottetown 80 and Hannchen.

Since O.A.C. 21 and Mensury make up the bulk of the six-rowed barley now sold for malting barley in Canada, it would appear that a satisfactory equation for prediction of the extract yield of commercial shipments of Canadian barley may be developed. Our data suggest that this will take the form:

$$\text{Malt extract} = 35.7 + 0.58 \text{ barley extract} - 2.4 \text{ total nitrogen}$$

However, as has been pointed out by Bishop (6, 7), the value of the varietal constant is affected by malting conditions. Thus our value, 35.7, based on studies of malts made in small-scale laboratory equipment, may not prove accurate for malts made in commercial malt houses.

General Prediction Equation

The possibilities of developing a general intervarietal prediction equation applicable at all stations appeared to merit investigation. For this purpose it is necessary to combine in one equation factors showing intervarietal and interstation relations with malt extract. Preliminary investigation suggested that the three properties, barley extract, steeping time, and total nitrogen were most promising. The following equation was developed from the 1937 data:—

$$\text{Malt extract} = 16.0 + 0.849 \text{ barley extract} - 0.043 \text{ steeping time} - 1.54 \text{ total nitrogen } (\pm 0.86)$$

The results of an analysis of the homogeneity of the relation, with respect to stations, are given in the first two columns of data in Table VIII. Significant differences between station regressions and between centroids were not established and the equation therefore seemed promising.

A reinvestigation of this equation was made with the 1938 data. The results of the analysis are shown in the last two columns of data in Table VIII. Once again our purpose was defeated since the analysis shows that for the 1938 data differences between centroids are significant. Accordingly, even in this equation, it would appear that separate constants for different stations should be employed.

TABLE VIII

ANALYSIS OF RESIDUAL VARIANCE FOR INTERVARIETAL RELATION BETWEEN MALT EXTRACT, BARLEY EXTRACT, STEEPING TIME, AND TOTAL NITROGEN

Variance due to:	1937 data		1938 data	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Differences among station regression coefficients	33	0.661	15	0.834
Deviations of centroids from average station regression	11	1.322	5	5.660**
Deviations from individual station regressions	96	0.729	120	0.738

A number of modifications of this equation were examined without success. Significant differences were invariably found between centroids. The authors consider it probable that adequate analyses of a sufficiently large body of data will invariably show that all general equations for the prediction of the malt extract yield of any sample of barley, irrespective of variety and origin, are theoretically unsound. It does not follow that a useful general equation cannot be developed but this will probably be based on compensating errors rather than on sound principles.

Discussion

The most useful intervarietal prediction equations for malt extract are based on the use of either barley extract or starch content. The explanation is readily apparent since it is obvious that a reasonably close relation must exist between malt extract and each of these two properties. Except for small portions, lost by respiration during malting and retained in the spent grains during mashing, all the starch in the barley is transformed into malt extract of which the starch degradation products form the predominant part. Similarly, barley extract, as determined in the present investigation, includes almost all of the barley constituents that are later transformed into extract, together with those that are lost during malting by steeping loss, respiration, and removal of sprouts. A close and fundamental relation between barley extract and malt extract would therefore be anticipated.

In effect it would appear that the determinations of barley extract provide a measure of the potentially extractable material in the barley kernel. Moreover, since the starch forms such a large proportion of this material, it also provides a measure of the potential extract. Thus both of these properties

provide a logical starting point for the development of a prediction equation for malt extract.

Having obtained a factor that measures the potential yield of malt extract, it would appear that the second factor required for the prediction of the actual yield should measure the degree to which the potential yield can be realized during the malting and mashing processes. This factor is probably associated with enzymatic activity and it can be assumed that the higher the enzymatic activity of the variety, the greater will be its yield of extract. However, two opposing reactions are involved. Increasing enzymatic activity will undoubtedly result in hydrolysis of increasing amounts of potentially extractable material. At the same time it also appears that increasing enzymatic activity will also result in increasing the malting loss resulting from the activity of the respiratory enzymes and the ready availability of soluble material for translocation to the roots which are subsequently discarded when the malt is polished. So far as can be determined from the results of the present investigation, the balance between these opposing reactions is favourable. Thus though increasing enzymatic activity results in a slight increase in malting loss, this appears to be more than offset by the additional hydrolysis of potentially extractable material. In this connection, it is interesting to note that an intervarietal association exists between malting loss and extract yield so that varieties that tend to have a higher malting loss also tend to produce a greater yield of extract.

The practical difficulty is that of discovering a barley property that provides an adequate measure of the total enzymatic activity of the grain. In this connection a determination of the saccharifying activity of a barley extract made in the presence of papain has been investigated (10). A fairly close intervarietal relation exists between this activity and the saccharifying activity of the finished malt. Moreover, between varieties, the saccharifying activity of the barley is associated to some extent with starch liquefying activity, autolytic diastatic activity, and proteolytic activity, which were the only other enzymatic properties investigated in this study (4, 11). Thus varieties that tend to be high in saccharifying activity of barley also tend to be high in proteolytic activity, autolytic diastatic activity, and starch liquefying activity of the malt. It would thus appear that saccharifying activity of barley might form an estimate of the total enzymatic activity developed during malting. Statistical studies show that this expectation could not be realized, although an improvement in the prediction of malt extract from starch content could be obtained by adding barley saccharifying activity as a second independent variable.

Although it is possible to obtain an estimate of the saccharifying activity of malt by making a determination of the saccharifying activity of the barley by means of a papain extract, there are not yet available, so far as we are aware, similar methods of estimating other enzymatic activities of malt. In the present study, the starch liquefying activity of the malt was measured directly. According to current hypothesis this activity is related to the content

of α-amylase and appears to be developed during the malting process. In consequence, it seems impossible to obtain an estimate of the starch-liquefying activity of malt by any type of determination made on the barley. In this study the proteolytic activity of the malt by an autolytic procedure was also determined. Although proteolytic activity increases very markedly during the malting process, the barley has some proteolytic activity prior to malting. Preliminary experiments, made to determine whether the proteolytic activity of the barley was related in any way to the proteolytic activity of the malt made from it, failed to suggest that any such relationship existed. The subject would appear to merit further investigation but must await the development of more satisfactory methods of estimating the activities of the various proteolytic enzymes.

Further statistical studies (11) indicated that the relations between barley saccharifying activity and the enzymatic activities of malt do not appear to represent fundamental relations. All these enzymatic activities are positively associated with salt-soluble barley nitrogen, and when the partial correlations between pairs of activities, independent of salt-soluble nitrogen, are computed, the coefficients fail to attain the 5% level of significance. It thus appears that the more fundamental relations involved are those between salt-soluble nitrogen and the enzymatic activities. Although these relations are not sufficiently close to permit accurate prediction of enzymatic activities of malt from the salt-soluble nitrogen of the barley, nevertheless, they suggest that a real relation exists between salt-soluble protein, or some fraction of it, and the enzymes present in barley and malt. Either the enzymes themselves are similar in nature to the salt-soluble proteins or these proteins act as carriers for the enzymes. It appears that salt-soluble barley nitrogen may well be a property that tends to represent total enzymatic activity of malt.

Confirmation of this hypothesis was obtained by a significant improvement in the prediction of malt extract when salt-soluble nitrogen was introduced as a second independent variable in equations involving barley extract or starch. In equations with starch and salt-soluble nitrogen an alternative hypothesis is possible. Since malt extract is composed largely of the hydrolytic products of starch and protein, it is not unreasonable to suppose that the salt-soluble protein may make a major contribution to the latter of these components. However, in equations involving barley extract it appears that the barley extract itself includes the major portion of the salt-soluble nitrogen in addition to the starch. Hence in these equations the beneficial effects of including salt-soluble nitrogen must depend on less direct relations, presumably those between this property and enzymatic activities.

A second property that has proved useful, in combination with barley extract or starch, for the prediction of malt extract, is steeping time. In the present investigation this represents the number of hours required for a barley to attain a moisture content of 46% when steeped under specified conditions of temperature and aeration. It is apparent at once that the usefulness of this property as a second independent variable must depend upon indirect

relations. Here again we are of the opinion that the relations in question are those between steeping time and individual enzymatic activities. Thus it has been shown that the intervarietal correlations between steeping time on the one hand, and saccharifying activity of barley, saccharifying activity, proteolytic activity, autolytic diastatic activity, and starch liquefying activity of malt on the other hand, all attain a 1% level of significance. The relations are all inverse so that varieties that tend to require a longer time in the steep tend to have lower enzymatic activities. The authors of this paper are not able to offer any satisfactory hypothesis to explain these associations. However, in this connection it should also be noted that there is a negative association between steeping time and salt-soluble nitrogen. Partial correlation coefficients, independent of steeping time, between the various pairs of enzymatic activities showed a close similarity to those independent of salt-soluble nitrogen. Hence it is concluded that the effectiveness of steeping time, in improving the prediction of malt extract from barley extract or starch, results from the associations of this property with the various enzymatic activities of the malt.

The investigation also showed that the intervarietal prediction of malt extract could be improved by including both salt-soluble nitrogen and steeping time with barley extract or starch. Here again the explanation appears to be that a combination of salt-soluble nitrogen and steeping time provides a better estimate of total enzymatic activities than either of these properties alone.

Turning now to the intravarietal (or interstation) prediction of malt extract, a rather different situation is found. As might be expected, barley extract still proves to be the most useful individual property for purposes of predicting malt extract. Again, we may assume that its utility depends primarily on the fact that it measures potentially extractable material. The same reasoning also appears to apply to the use of total nitrogen as a prediction factor. Its utility has been widely investigated by Bishop who suggests that it provides an inverse measure of the potentially extractable carbohydrate material (8).

Following the line of reasoning used in discussing the intervarietal prediction of extract, it would appear that in intravarietal prediction a search should also be made for a second factor representing the degree to which the potentially extractable material can be made available. Certain obvious difficulties are at once faced. It has been demonstrated conclusively that a change in environment, which results in an increase in barley extract or starch, also results in a decrease in total nitrogen content (13) and, with the exception of autolytic diastatic activity (3, 4, 11), of all enzymatic activities so far investigated. Thus in considering the effect of environment on malt extract we are dealing with two opposing tendencies, an increase in potentially extractable material associated with a decrease in enzymatic activities. A third factor may also play a part in this connection. It is quite possible, for instance, that the degree to which potentially extractable material can be realized during the malting

and mashing process depends not only on the activity of enzymes involved but also on the facility with which the extractable material can be attacked by these enzymes. Bishop (8) has put forward an hypothesis in this connection in which he suggests that a decrease in malt extract is associated with an increase in total nitrogen content not only because an increase in proteins must result in the displacement of extractable carbohydrates but also because there is a tendency for some potentially extractable carbohydrates to be "locked up" by the proteins.

The only factor we have discovered that will improve the intravarietal prediction of malt extract from barley extract, is total nitrogen. Since in the prediction equation the partial regression coefficient for total nitrogen on malt extract is negative, there is no reason to believe that the improvement resulting from the inclusion of total nitrogen as a second independent variable is related to the association that exists between total nitrogen and enzymatic activities. If this hypothesis were correct, the partial regression coefficient should be positive rather than negative. Accordingly, it appears that in considering this relation between malt extract on the one hand, and barley extract and total nitrogen, on the other hand, we are forced to conclude that the effect of the last named factor is related to the degree in which total nitrogen "seals up" potentially extractable barley materials.

As was pointed out earlier in this paper, there appears to be little possibility of developing a general equation that will be satisfactory for both the inter- and intravarietal prediction of malt extract. Such an equation can only be be developed if two sets of conditions prevail. Firstly, if it is possible to find factors such as barley extract and starch which are related to malt extract both within and between varieties, and secondly, if the regressions of these properties on malt extract are identical both within and between varieties. It has not been possible to find any properties that meet the second of these conditions. The inter- and intravarietal regression coefficients invariably differ appreciably. This might well be expected since no single barley property has yet been found that is sufficiently closely related to malt extract to provide adequate prediction by itself. Malt extract may be dependent principally upon the potential yield of extractable materials existing in the barley as measured by barley extract or starch, but it is also conditioned by a variety of other factors that appear to act differently within and between varieties. In consequence, the regressions of malt extract on barley extract or starch are not identical within and between varieties. Theoretically, it is quite possible that two or three barley properties might be found that are related to malt extract both in an inter- and intravarietal manner. If a prediction equation based on these properties were then developed it might finally appear that the partial regressions of each property on malt extract were identical both within and between varieties. It must be admitted, however, that our practical attempts to solve this theoretically soluble problem have not proved successful.

In surveying the results of the study presented in this paper, the authors are forced to admit that they do not expect that wide use will be made of the

various prediction equations discussed above. Prediction equations are, after all, only means of expressing the relations that exist between various barley properties and malt extract. They do not fail to be useful merely because they are not used. They may well serve to bring to light the various associations and more fundamental relations that exist between barley and malt properties, and may thus help to elucidate the nature of malting quality in barley. A clear conception of underlying principles is surely a necessary prerequisite to the development of a logical program designed to produce new varieties of improved agronomic quality and satisfactory malting quality.

The practical advantages that arise from a detailed knowledge of the relations between malt extract and barley properties are two, and these both depend upon an extensive knowledge of the chemical characteristics of the parent materials used in the breeding program. Firstly, it should be possible to select pairs of parents that are complementary in that they are not characterized by the same faults. Secondly, it should be possible, usually by the use of a single determination made on a small amount of barley, to select from the progeny of certain crosses those lines that are not characterized by an important malting deficiency. In Canada, close co-operation already exists between plant breeders and those engaged in laboratory studies of malting quality. It is hoped that as a result of the studies reported in this series of papers, laboratory workers will now be in a position to give more extensive and useful aid to plant breeders.

References

1. Anderson, J. A. and Ayre, C. A. Can. J. Research, C, 16 : 377-390. 1938.
2. Anderson, J. A., Ayre, C. A., and Meredith, W. O. S. Can. J. Research, C, 17 : 25-34. 1939.
3. Anderson, J. A., Sallans, H. R., and Ayre, C. A. Can. J. Research, C, 16 : 456-466. 1938.
4. Ayre, C. A. and Anderson, J. A. Can. J. Research, C, 17 : 239-246. 1939.
5. Ayre, C. A., Sallans, H. R., and Anderson, J. A. Can. J. Research, C, 18 : 169-177. 1940.
6. Bishop, L. R. J. Inst. Brewing, 36 (8) : 336-349. 1930.
7. Bishop, L. R. J. Inst. Brewing, 36 (9) : 421-434. 1930.
8. Bishop, L. R. J. Inst. Brewing, 40 (3) : 75-91. 1934.
9. Bishop, L. R. J. Inst. Brewing, 42 (1) : 10-14. 1936.
10. Sallans, H. R. and Anderson, J. A. Can. J. Research, C, 16 : 405-416. 1938.
11. Sallans, H. R. and Anderson, J. A. Can. J. Research, C, 17 : 361-372. 1939.
12. Sallans, H. R. and Anderson, J. A. Can. J. Research, C, 18 : 35-41. 1940.
13. Sallans, H. R. and Anderson, J. A. Can. J. Research, C, 18 : 219-229. 1940.

STUDIES ON FILM-FORMING YEASTS[1]

I. MEDIA AND METHODS

By V. E. Graham[2] and E. G. Hastings[3]

Abstract

The methods used for the isolation and study of film-forming yeasts are described. Two of these methods are of special interest, viz., the observation of the structure of pseudomycelium on "cross-hatched" plates and the preparation of gypsum cultures in test tubes.

Introduction

Certain yeast-like organisms produce a film of varying thickness on the surface of liquid media and on foods in which ethyl alcohol and some of the common organic acids are present in moderate concentration. Because of their film-forming character such organisms are often referred to as "Mycoderma". This term has, however, been used as a generic name for many years, and a continuation of its use as a descriptive term leads to confusion. In this work the term "film-forming yeasts" is used in referring to the group as a whole and the term *Mycoderma* is used only in referring to the organisms belonging to this genus as described by Lodder (6).

In this study, film-forming yeasts have been isolated from rennet brine, Swiss cheese brine, ensilage, butter, and pickles. This paper deals with the methods that were used in the study of these organisms. The types found in the products mentioned will be discussed in later reports.

The literature dealing with the classification of the film-forming yeasts has been summarized elsewhere (3). This literature is voluminous but much of it is now only of historical interest. The nomenclature followed in this study is essentially that of Stelling-Dekker (8), Lodder (6), and Diddens and Lodder (2).

Media and Methods

Most of the methods used in this study of film-forming yeasts are familiar bacteriological and mycological procedures and will not be discussed in detail. The methods used for isolation and for the study of carbon and nitrogen sources will be presented, together with some observations on the problems of pseudomycelium formation and spore formation.

Isolation of Film-forming Yeasts

Film-forming yeasts may be isolated directly by ordinary plating on acid wort or malt agar when they constitute an appreciable proportion of the flora in the substance in which they occur. The enrichment procedure is the

[1] *Manuscript received in original form, July 11, 1940, and as revised, April 28, 1941.*

[2] *Professor of Dairying, University of Saskatchewan, Saskatoon, Sask.*

[3] *Chairman, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wis., U.S.A.*

more generally useful one and was used in this work prior to plating. The composition of three useful enrichment media is given below.

Enrichment Medium No. 1

Bacto peptone	0.5%
Absolute alcohol	0.5%
Sodium chloride	0.5%

The reaction was adjusted to pH 4.6 by the addition of lactic acid.

Enrichment Medium No. 2

Magnesium sulphate	0.02%
Dipotassium phosphate	0.10%
Bacto peptone	0.50%
Glacial acetic acid	0.40%

The reaction was not adjusted.

Enrichment Medium No. 3

Ammonium sulphate	0.25%
Dipotassium phosphate	0.05%
Magnesium sulphate	0.01%
Bacto peptone	0.40%
Absolute alcohol	1.00%

The reaction was adjusted to pH 3.5 by the addition of lactic acid. The alcohol was usually added before sterilization but the concentration may be more closely regulated if it is added afterwards.

The media were placed in 6-oz. bottles to a depth of about 5 cm. and were plugged with cotton to allow free access of air. They were sterilized in the autoclave at 15 lb. pressure for 25 min. The medium was inoculated with some of the material from which the isolation of film-forming yeasts was sought. When a film had formed, a portion of it was transferred to another bottle of similar medium. If another film formed and microscopic examination showed the presence of yeast-like organisms in large numbers, loop dilution plates were prepared on acid glucose agar and the isolation and purification of the organisms was completed in the usual manner.

The medium containing acetic acid was devised for the isolation from soil of yeasts that could utilize this acid and also to aid in controlling mould growth. Medium No. 3 proved to be more generally useful than the others but No. 1 was used successfully in the isolation of *Debaryomyces* from rennet brine.

The principle of enrichment is very useful in securing cultures of film-forming yeasts. A variety of media can be devised for this purpose depending upon the growth requirements of the group being sought. Liquid cultures should not be disturbed; shaking causes the film to sink and tends to inhibit the growth of film-forming organisms.

Nitrogen Sources

Liquid media were used in studying the availability of nitrogen from ammonium sulphate, potassium nitrate, and peptone. The basic medium was that used by Stelling-Dekker (8), viz.:

Glucose	2.00%
Monopotassium phosphate	0.10%
Magnesium sulphate	0.05%

The nitrogen sources were added in the following proportions by weight: ammonium sulphate, 0.25%; potassium nitrate, 0.10%; peptone, 0.5%. Controls were used from which the nitrogen source was omitted. Distilled water must be used in preparing these media.

Carbon Sources

A determination of the materials that can be used as sources of carbon is often useful in classifying micro-organisms. The ability of an organism to secure carbon from a particular compound can be determined readily if it can utilize inorganic salts as nitrogen sources. For this purpose the following basic medium gave satisfactory results.

Monopotassium phosphate	0.10%
Magnesium sulphate	0.05%
Ammonium sulphate	0.25%

The carbon sources studied were added to this basic medium in the following proportions by weight: glucose, 1 or 2%; sucrose, lactose, and maltose, 1%; valeric, caprylic, and capric acids, 0.5%; butyric and caproic acids, 1%; maleic and tartaric acids, 0.33%; citric acid as 0.5% sodium citrate. In studying growth on ethyl alcohol 4% by volume was added after sterilization. All media were sterilized by autoclaving at 15 lb. pressure for 25 min.

When an organism cannot use inorganic compounds as nitrogen sources, peptone must be added to the medium. Since there is then growth on the control tubes, this somewhat complicates the study of carbon sources. In such cultures the decision regarding utilization was based on observation of the relative abundance of film formation on the control tube and the tube containing the added sugar or acid, and also by the final acidity or alkalinity. If only the peptone was used as a source of carbon the medium usually became alkaline, whereas if the sugar or other source of carbon was consumed, some acid was usually produced.

Stelling-Dekker (8) and Lodder (6) restrict the use of the term "fermentation" to the utilization of a sugar with the production of gas. "Utilization", on the other hand, was used to indicate that the organism consumed the sugar as a carbon source, but without gas production. In this work these terms are used in the limited sense.

Other Media

Cultures were carried on yeast water agar containing 2% glucose. Wort and malt agar are also satisfactory for this purpose and were used to some

extent. These and other media to which reference is made are commonly known and will not be described.

The Detection of Pseudomycelium

Lodder (6) has defined a true mycelium as one that is non-septate, or one in which the septa have been formed *within* the filament; a pseudomycelium on the other hand, is one that has been formed by the budding of its member cells, the septa thus being formed from the outside. This distinction is reasonably clear, although there are fungi in which both types of mycelium may be present. Pseudomycelium formation is an important character to be considered in the classification of yeast-like organisms and the conditions that favour its production have been studied in detail by many workers. It is important to recognize that the firmness with which the cells constituting the pseudomycelium are held together varies with different yeasts. Consequently, if the structure of the pseudomycelium is to be observed accurately, the method employed should be one in which the cells may be seen in the position in which they have developed. These facts explain the popularity of the slide culture technique of Rivalier and Seydel (7) and its various modifications (3).

It was found in the present study that pseudomycelium formation may be observed readily on an ordinary cross-hatched plate. Such plates were prepared by pouring 2% glucose yeast water agar in Petri dishes to a depth of about 6 mm. and inoculating by crossed streaks about 2 cm. apart as soon as the agar had hardened. The pseudomycelium grows out on the moist plate without distortion. The growth on such a plate is most abundant at the ends of the streaks and toward the periphery of the dish; it is least abundant toward the centre. Pseudomycelium formation is readily seen along the edges of the inner streaks in which growth is restricted. A detailed study of strands of the pseudomycelium may be made by placing mineral oil on the agar at the edge of a streak and, as described by Dalmau (1), examining directly with the oil immersion objective. This method requires no special medium or equipment and gives excellent results.

Spore Formation

The formation of ascospores is one of the most valuable characters to be considered in the classification of yeasts and many methods have been described for inducing these organisms to form spores. A study of the results of many workers indicates that the requirements for sporulation are not necessarily the same for all genera. As a rule the conclusion of Heinz (5) that conditions that favour vegetative growth tend to inhibit spore formation seems to be true; an exception, however, is found in at least one species of *Pichia*.

Three of the most commonly used methods for the detection of spore formation are: cultivation on gypsum blocks, carrot slices, and Gorodkowa agar. All these methods were used in the present study. Gorodkowa agar

has given excellent results in studies on the genera *Hansenula* and *Debaryomyces*.

A New Method of Preparing Gypsum Cultures

Preparing gypsum cultures by Hansen's method (4) involves the expenditure of much time and labour; also the cultures tend to dry out and become contaminated. When it became necessary to use gypsum cultures in rather large numbers in this study the idea presented itself of making gypsum slants in ordinary test tubes. Preliminary experiments demonstrated that this procedure was feasible and that, apart from eliminating much of the work and difficulty ordinarily involved in the preparation of such material, the method was also easier and the tubes were less liable to contamination than dish cultures. The procedure developed is as follows. Procure a sufficient number of clean test tubes, preferably of the 18 by 150 mm. size. Set up a filling funnel equipped with rubber tubing and a stopcock but without a glass tip on the filling tube. The end of the tubing should be long enough to reach to the bottom of the test tube. Mix gypsum with water in such proportions that the mixture is of the consistency of thick cream. Fill the tubes in the ordinary manner but run the end of the filling tube down to within an inch of the bottom of the test tubes. If the gypsum has been mixed to the proper consistency there will be no drip from the end of this filling tube when the pinchcock is closed and thus there will be no drops of gypsum on the side of the test tube. Fill the tubes to a depth of about 1 in., place in suitable racks, and slope them to give a long surface for inoculation. Place the rack in this sloping position in the incubator at about 50° C. to harden. This will require 24 to 48 hr. When the slopes are hard, remove from the incubator, plug the tubes with cotton, and sterilize in the autoclave. Thorough hardening is important; if the gypsum is not properly dried, it will swell during autoclaving, owing to the formation of steam within the mass, and the tubes are useless.

Gypsum slopes prepared in this manner will keep indefinitely if given proper protection. When a culture is to be prepared, some moisture must be added to the tubes. This may be in the form of sterile water, wort, phosphate solution, or any other type of nutrient desired. About 3 ml. of the solution is usually required.

The tubes may be cleaned by filling with water, allowing to soak overnight, and then breaking the block loose from the tube with a small rod. The water tends to creep between the gypsum and the glass and the whole block usually slips out readily.

These tubes are handled in exactly the same manner as agar slants and have many obvious advantages over the usual type of dish culture. This method makes gypsum culture feasible on a large scale, either for research or teaching purposes.

References

1. DALMAU, L. M. Ann. Parasitol. 7 : 536-545. 1929.
2. DIDDENS, H. A. and LODDER, J. Mycopathologia, 2 : 1-6. 1939.
3. GRAHAM, V. E. Thesis, Univ. Wisconsin. 1939.
4. HANSEN, E. C. Résumé. Compt. rend. trav. lab. Carlsberg, 2 : 13-47. 1883.
5. HEINZ, W. Centr. Bakt. Parasitenk. 44 (Abt. 2) : 184-185. 1916.
6. LODDER, J. Verhandel. Akad. Wetenschappen, Amsterdam, 2nd Sect. 32:1-256. 1934.
7. RIVALIER, E. and SEYDEL, S. Compt. rend. soc. biol. 110 : 181-184. 1932.
8. STELLING-DEKKER, N. M. Verhandel. Akad. Wetenschappen, Amsterdam, 2nd Sect. 28:1-547. 1931.

VEGETATIVE PROPAGATION OF CONIFERS

IX. EFFECTS OF CHEMICAL TREATMENTS AND A WAX SPRAY ON THE OUTDOOR PROPAGATION OF SPRUCE CUTTINGS[1]

By N. H. Grace[2] and J. L. Farrar[3]

Abstract

Norway spruce cuttings were collected at intervals throughout the year, subjected to treatment with talc dusts containing from 5 up to 10,000 p.p.m. of indolylacetic acid, and propagated in outdoor frames. In some experiments, indolylacetic acid treatments were included in a series of dusts involving cane sugar, potassium acid phosphate, and organic mercurial disinfectants. Effects of wax coating of cuttings of Norway and white spruce and eastern white cedar were also considered.

Indolylacetic acid treatment failed to have any general beneficial effects on rooting; concentrations of 8000 and 10,000 p.p.m. were usually injurious. However, treatment of apparently dormant cuttings, taken shortly before emergence of new growth, with 1000 p.p.m. gave 25% rooting, as compared to 8% for the controls, and tended to increase the length of root. Treatments with organic mercury, cane sugar, and potassium acid phosphate increased survival of new growth, and, in combination with indolylacetic acid, increased survival and root lengths.

Talc treatment increased rooting to as much as 70% for cuttings planted in sand as compared to 25% for the controls, but had no effect on plantings in a sand–peat mixture. Talc effects were the most marked on cuttings taken before emergence of new growth and when planting was delayed for 24 hr. after treatment.

Wax application had no effect on dormant spruce cuttings but was markedly injurious to summer collections. Injurious effects were reduced when wax was used in conjunction with indolylacetic acid treatment.

Introduction

The effects of a number of chemical treatments on the responses of conifer cuttings have been reported (2–4, 6, 7, 9, 10, 14–20, 22, 24, 25, 29, 32). Early communications by the authors dealt with greenhouse propagation (3, 4, 6, 7, 9, 10, 14, 18). Since most applications of vegetative propagation to forestry necessitate large-scale operations, the use of outdoor frames has been given some consideration (5, 14, 17). This paper describes the effects of chemical treatments on cuttings propagated outdoors as shown by 16 experiments involving 16,650 cuttings.

Experimental

Materials

All spruce cuttings were from 6 to 10 cm. long and were made of the full length of the current year's growth, in most cases from the lower branches of the tree. Norway spruce (*Picea Abies* (L.) Karst.) cuttings were obtained from a plantation 19 years old at the Petawawa Forest Experiment Station,

[1] *Manuscript received April 18, 1941.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa, and the Dominion Forest Service, Ottawa. Part of a co-operative project of the Subcommittee on Forest Tree Breeding, Associate Committee on Forestry, N.R.C. No. 997.

[2] *Biochemist, National Research Laboratories.*

[3] *Forestry Engineer, Dominion Forest Service, Ottawa.*

Chalk River, Ontario. Collections were taken at monthly intervals from November to April, at several periods in May and June just prior to, and during, the emergence of new growth, and at bimonthly intervals from July to October.

Cuttings of the winter collections, in groups, were heeled in and put outdoors in flats till May when they were lined out in the frames. Those of the other collections were planted outdoors directly. Some experiments were done in sand and the others in sand–peat.

Effects of a wax emulsion were determined on white spruce (*Picea glauca* (Moench) Voss.) and eastern white cedar (*Thuja occidentalis* L.) as well as Norway spruce. These cuttings were obtained in Ottawa from planted trees about 15 years old. Spruce cuttings were collected while apparently dormant, about the time of emergence of new growth, and at intervals during the summer. Cedar cuttings were taken early in May while still dormant.

Treatment of Cuttings

The chemicals were applied to the cuttings in finely ground talc, since the authors have found the solution method of treatment to be injurious or without effect (3, 4, 14, 18). The basal two centimetres of the cuttings were dusted without preliminary moistening and planted within two hours, except in one case where delayed planting was being investigated.

A phytohormone chemical was used in all experiments. Indolylacetic acid was selected because some beneficial effects on conifer cuttings had already been obtained through its use (6, 10, 25, 32). It was mixed with talc in concentrations of 5 to 10,000 p.p.m. (parts of the chemical per million parts o the mixture by weight), though in most experiments the concentrations were 5, 100, 1000, and 2000 p.p.m.

Most experiments were of a factorial design permitting the examination of interaction effects between phytohormone treatments and other factors such as the developmental stage of the tree, the position of the cutting on the tree, type of cutting, delayed planting, and media.[1] Only one or two of these factors were combined with indolylacetic acid treatment in any one experiment. Groups of untreated and talc treated cuttings were used as controls.

Nutrient and disinfectant chemicals were used in two experiments which are numbered and described in detail because they relate to a series of experiments dealing with the effects of relatively complex dust mixtures on cuttings of Norway spruce and other plants (8, 10, 13, 17).

Experiment 1

Cuttings of Norway spruce were collected from both the upper and lower thirds of the tree, May 17, 1939, while the buds were still dormant, and were treated in groups of 12 with a series of eight dusts (10). The series involved

[1] *The primary effects of these factors will be reported in later papers.*

indolylacetic acid at concentrations of 0 and 1000 p.p.m. alone, and in combination with cane sugar at 0 and 10% and ethyl mercuric phosphate at 0 and 10 p.p.m. There were four replicate blocks in the experiment making a total of 768 cuttings.

Experiment 2

Cuttings of Norway spruce bearing an opening bud with a new shoot ranging from 0.5 to 1 cm. in length, were collected from the lower third of the tree on June 9, 1939. Groups of 10 cuttings were treated with a series of 32 dusts containing indolylacetic acid, potassium acid phosphate, cane sugar, and ethyl mercuric bromide. Effects of the series of dust treatments on both greenwood and dormant cuttings have been previously described (13). There were six replicates of the 32 treatments; three replicates were sprayed with wax. The experiment required 1920 cuttings.

The wax emulsion[1] is one designed to reduce transpiration and is recommended for use with conifer transplants. The emulsion, diluted with two volumes of distilled water, was sprayed over the entire cutting with the exception of the basal two centimetres which were covered during application. Groups of cuttings were held for 30 min. before planting to permit the wax to harden.

Arrangement of Experiments

All experiments were arranged according to the principles of experimental design with treatments replicated and the groups randomized in the propagating frames (28). The data were analysed by the analysis of variance procedure as illustrated by Tables I and II. Data on numbers of cuttings were subjected to the inverse sine transformation prior to analysis (1).

Observations

Cuttings were removed approximately one year after planting and record was taken of the number of cuttings surviving, callused, rooted, bearing new growth, with roots and new growth, and the number and length of roots. The number and length of roots per rooted cutting and the mean root length could be calculated from these data. Approximately four months after the spring plantings, record was made of the number of cuttings surviving and with living new growth.

Results

The results of Experiments 1 and 2, dealing chiefly with nutrient and disinfectant chemicals, are described in detail. The results of the other experiments are summarized to demonstrate the effects of treatment.

Experiment 1

Effects of Indolylacetic Acid, Cane Sugar, and Ethyl Mercuric Phosphate

Cane sugar and organic mercury treatment each increased rooting in the absence of the other; in combination there was no beneficial effect.

[1] *Dowax, purchased from the Dow Chemical Company, Midland, Michigan, U.S.A.*

TABLE I

EXPERIMENT 2. ANALYSIS OF VARIANCE OF RESPONSES OF NORWAY SPRUCE CUTTINGS TREATED WITH TALC DUSTS CONTAINING INDOLYLACETIC ACID, POTASSIUM ACID PHOSPHATE, CANE SUGAR, AND ORGANIC MERCURY AND SPRAYED WITH A WAX EMULSION

Source of variance	Degrees of freedom	Mean square			
		Number of cuttings			
		Rooted	Alive, not rooted	Living, new growth	Dead, new growth
Replicates	2	2362.8***	585.1*	603.0	181.5
Treatments—					
Indolylacetic acid	1	94.9	641.7	619.9	354.8
Organic mercury	1	780.1	5.7	94.9	5.7
Phosphate	3	117.4	754.1**	1320.1***	655.4**
Cane sugar	1	223.2	478.2	24.8	20.7
Interactions—					
Indolylacetic acid × organic mercury	1	141.8	641.7	5.7	86.7
Indolylacetic acid × phosphate	3	201.9	176.4	189.1	28.4
Indolylacetic acid × cane sugar	1	223.2	1.2	1068.8*	131.7
Organic mercury × phosphate	3	63.6	11.7	32.0	229.6
Organic mercury × cane sugar	1	405.4	24.8	379.7	1931.7***
Phosphate × cane sugar	3	253.9	63.4	413.4	461.8*
Indolylacetic acid × organic mercury × phosphate	3	81.6	76.2	603.6*	285.6
Indolylacetic acid × phosphate × cane sugar	3	58.2	62.4	22.2	42.3
Indolylacetic acid × organic mercury × cane sugar	1	5.7	186.0	143.4	391.2
Organic mercury × phosphate × cane sugar	3	173.4	176.8	282.1	128.6
Indolylacetic acid × organic mercury × phosphate × cane sugar	3	123.5	141.2	116.6	351.6
Error *(a)*	62	213.9	165.5	213.4	158.1
Waxed *vs.* unwaxed	1	41506.9***	5260.5***	5775.1***	4890.4***
Interactions treatments × wax—					
Indolylacetic acid × wax	1	1068.8*	64.2	2047.5**	2458.2***
Organic mercury × wax	1	121.9	152.3	0.4	0.4
Phosphate × wax	3	92.7	376.6	57.9	245.1
Cane sugar × wax	1	459.4	263.7	756.1*	307.6
Indolylacetic acid × organic mercury × wax	1	13.6	20.7	7.9	34.0
Indolylacetic acid × phosphate × wax	3	89.8	7.7	155.7	29.3
Indolylacetic acid × cane sugar × wax	1	247.7	0.1	423.0	10.5
Organic mercury × phosphate × wax	3	343.9	159.6	12.3	242.8
Organic mercury × cane sugar × wax	1	598.6	263.7	1040.7*	441.1
Phosphate × cane sugar × wax	3	79.2	56.2	333.2	102.2
Indolylacetic acid × organic mercury × phosphate × wax	3	118.3	12.9	226.1	182.1
Indolylacetic acid × phosphate × cane sugar × wax	3	69.0	42.1	163.9	101.1
Indolylacetic acid × organic mercury × cane sugar × wax	1	1.2	1.2	1.2	3.8
Organic mercury × phosphate × cane sugar × wax	3	175.6	249.1	126.8	109.8
Indolylacetic acid × organic mercury × phosphate × cane sugar × wax	3	282.4	176.4	297.1	405.8*
Error *(b)*	64	177.7	215.8	185.6	129.5

* *Exceeds mean square error, 5% level of significance.*
** *Exceeds mean square error, 1% level of significance.*
*** *Exceeds mean square error, 0.1% level of significance.*

TABLE II

EXPERIMENT 2. ANALYSIS OF VARIANCE OF RESPONSES OF NORWAY SPRUCE CUTTINGS TREATED WITH TALC DUSTS CONTAINING INDOLYLACETIC ACID, POTASSIUM ACID PHOSPHATE, CANE SUGAR, AND ORGANIC MERCURY

Source of variance	Degrees of freedom	Mean square		
		Number of roots per rooted cutting	Length of root per rooted cutting	Mean root length
Replicates	2	0.75	678.5	678.5**
Treatments—				
Indolylacetic acid	1	.01	145.0	12.0
Organic mercury	1	.18	0.2	135.4
Phosphate	3	.96	1176.2	169.7
Cane sugar	1	.47	1944.0	9.4
Interactions—				
Indolylacetic acid × organic mercury	1	1.28	181.5	3.4
Indolylacetic acid × phosphate	3	0.72	464.8	68.6
Indolylacetic acid × cane sugar	1	.01	5766.0*	495.0
Organic mercury × phosphate	3	.19	2423.6	468.9*
Organic mercury × cane sugar	1	.00	2147.0	92.0
Phosphate × cane sugar	3	.20	316.5	67.4
Indolylacetic acid × organic mercury × phosphate	3	.37	1997.1	332.7
Indolylacetic acid × phosphate × cane sugar	3	1.07	5525.7**	419.1*
Indolylacetic acid × organic mercury × cane sugar	1	0.01	1162.0	234.4
Organic mercury × phosphate × cane sugar	3	.29	199.2	103.1
Indolylacetic acid × organic mercury × phosphate × cane sugar	3	.83	37.6	161.7
Error	62	.43	943.6	132.2

* *Exceeds mean square error, 5% level of significance.*
** *Exceeds mean square error, 1% level of significance.*

Results for rooting and survival indicated similar interactions between indolylacetic acid and cane sugar treatments, each being beneficial in the absence of the other. In the absence of sugar, indolylacetic acid, on the average, gave 25% rooting as compared to 8% for the controls. Early survival counts indicated beneficial effects from indolylacetic acid and cane sugar treatments on upper but not on lower cuttings. Conversely, the combination of organic mercury and indolylacetic acid increased survival in lower but not upper cuttings.

EXPERIMENT 2

EFFECTS OF POTASSIUM ACID PHOSPHATE, CANE SUGAR, ETHYL MERCURIC BROMIDE, INDOLYLACETIC ACID, AND A WAX SPRAY

In Table I are given the results of statistical treatment of data for the number of cuttings rooted, the number alive but not rooted, and the number with living and dead new growth; the data for the last two items were taken in late summer after the cuttings had been four months in the bed. Similar

results are given in Table II for the numbers and lengths of roots per rooted cutting and the mean root length; these results refer only to the unwaxed cuttings. It is apparent that there were numerous significant primary effects and interactions, and the more important of these are discussed.

Number of Rooted and Surviving Cuttings

Average rooting was 22% for the waxed and 64% for the unwaxed cuttings. Indolylacetic acid treatment gave 25% rooting for waxed cuttings as compared to 18% for the controls but was without effect on the unwaxed. At the end of the experiment 35% of the waxed and 21% of the unwaxed cuttings were alive but not rooted; the corresponding mortality was 44% and 15%. The data in Table III indicate that all concentrations of phosphate tended to reduce the number of living non-rooted cuttings.

Number of Cuttings with New Growth

The early survival count of new growth indicated that 16% of the cuttings had lost the opening bud. Wax treatment resulted in 28% of cuttings with living and 55% with dead new growth. Corresponding percentages for the unwaxed were 15 and 70. The data of Table III indicate that phosphate treatment tended to increase survival of new growth.

TABLE III

EXPERIMENT 2. AVERAGE EFFECTS OF POTASSIUM ACID PHOSPHATE ON NORWAY SPRUCE CUTTINGS

	Potassium acid phosphate in talc, %				Necessary difference, 5% level
	0	0.1	1	10	
Number of cuttings living, not rooted					
Transformed data	34.6	31.2	27.9	25.5	5.3
Per cent	36.7	29.8	25.8	19.6	
Number of cuttings, with living new growth					
Transformed data	19.7	21.4	21.9	31.3	6.0
Per cent	17.3	18.5	19.2	30.0	
Number of cuttings, with dead new growth					
Transformed data	56.1	55.2	54.2	47.9	5.1
Per cent	67.1	66.3	63.3	54.2	

Indolylacetic acid treatment decreased the number of waxed cuttings with living new growth but had no effect on the unwaxed. Cane sugar and organic mercury treatments, on the average, increased the survival of new growth in the absence of wax treatment. Combination of organic mercury with the 10% concentration of phosphate was beneficial in the presence of indolylacetic acid. Both cane sugar and organic mercury treatments tended to increase mortality of new growth, but when in combination there was no injurious effect. Cane sugar and phosphate treatments decreased mortality, but their combination effected no further decrease.

Final examination revealed that only 12% of the cuttings had living 1939 growth. About 4% of the cuttings had 1940 growth, slightly less than half of which came directly from 1938 wood, 1939 growth being absent. Practically all cuttings with 1940 growth were rooted, but a number of cuttings with living 1939 growth were not rooted; this was the more marked with waxed cuttings. Only 23% of the rooted cuttings had living new growth.

Number and Length of Roots per Rooted Cutting

The average number of roots per rooted cutting was 3.1, the average length, 11.1 cm. Data for the interaction effects of indolylacetic acid and cane sugar treatments on the length of root are given in Table IV. Whereas each chemical separately, on the average, tended to increase root length, the combination reduced length. The 10% phosphate concentration increased root length when in combination with either indolylacetic acid or cane sugar, but not with both.

TABLE IV

EXPERIMENT 2. AVERAGE EFFECTS OF INDOLYLACETIC ACID AND CANE SUGAR TREATMENT ON THE LENGTH OF ROOT PER ROOTED CUTTING, CM.

Indolylacetic acid in talc, p.p.m.	Cane sugar in talc, %	
	0	10
0	10.9	11.5
1000	12.2	9.7

Necessary difference, 5% level of significance: 1.8.

Mean Root Length

The mean root length averaged 3.7 cm. The combination of organic mercury and the 10% phosphate concentration resulted in increased length, though the chemicals were somewhat more effective when used separately. The interaction between the 10% phosphate concentration, indolylacetic acid, and cane sugar was similar to that discussed for length of root per rooted cutting.

EFFECTS OF TALC TREATMENT

Talc treatment increased rooting of spring and early summer collections of Norway spruce cuttings propagated in sand. Rooting of cuttings from the lower portions of the tree taken in early May was 23% after talc treatment, as compared to 4% for the controls; upper cuttings failed to show any increase. Early June collections, taken just as new growth was breaking on the cuttings, gave 70% rooting, as compared to 25% for the controls. Summer collections of new growth taken in July and August responded favourably to talc treatment; rooting was increased from 13% for the controls to 40%. Talc treatment effected some increases in root length, particularly with cuttings taken just as new growth was developing. At other seasons of the year, or when

propagation occurred in sand–peat mixture, talc treatment had no effect on rooting, though in some instances there were increases in the length of root per rooted cutting. Delay of 24 hr. in planting after preparation and treatment of cuttings was followed by beneficial effects from talc treatment.

Effects of Indolylacetic Acid Treatment

Indolylacetic acid treatment in some cases suggested beneficial effects in the number rooted and the number and lengths of roots when the cuttings were taken just prior to the emergence of new growth and propagated in sand. It also increased rooting of waxed cuttings (Experiment 2). In sand–peat mixtures and in the other experiments in sand, indolylacetic acid was not beneficial. Sometimes it was damaging, almost always when used in concentrations of 8000 and 10,000 p.p.m.

Effects of Waxing

The application of wax to Norway and white spruce cuttings had no effect on rooting or mortality during dormancy. It was injurious to cuttings with an emerging shoot, and to greenwood cuttings. Injurious effects of wax treatment on eastern white cedar cuttings were restricted to untreated and talc treated cuttings. In combination with 5, 100, and 1000 p.p.m. of indolylacetic acid, waxing had no adverse effects on mortality.

Discussion

Phytohormone, nutrient, and disinfectant chemicals, applied as dusts, have been shown to affect the responses of Norway spruce cuttings. The beneficial effects have been of small magnitude, and the same chemical may be beneficial, without effect, or damaging, depending on conditions. Hitchcock and Zimmerman (19) have emphasized that many conditions, e.g., age and relative activity of the shoot, time of year, and method of applying the substance, affect the response of cuttings to chemicals. Komissarov (25) and Kirkpatrick (23) have both pointed out that high temperatures are necessary for root-inducing substances to be effective. This is contrary to our experience in which cuttings treated in November and left outdoors in subzero temperatures over winter responded very favourably to treatment (17) and cuttings treated in summer did not respond at all. This is not to be taken as a contradiction but merely an illustration of the importance of other factors relating to the environment and the nature of the cuttings themselves in obtaining a response to treatment.

In the example just mentioned (17) the response to treatment with indolylacetic acid was modified by the medium in which the cuttings were grown. Experiments herein reported show that the part of the tree from which the cuttings are taken also affects their response to treatment.

Although indolylacetic acid is undoubtedly advantageous under certain conditions, its use in the propagation of Norway spruce does not seem to be warranted, at least until a number of other factors become better understood.

The beneficial results from talc treatment are in agreement with earlier findings dealing with conifers and other plants (11, 12, 14, 19, 21, 27, 30). The favourable response to talc when planting had been delayed, and when the cuttings were put in a relatively dry medium, sand, corroborates the suggestion of Hitchcock and Zimmerman (19) that talc affects the water relations of the cuttings. Damage due to talc treatment was observed in some experiments and has been previously reported (14). This variability is no doubt another illustration of conditions affecting the response to treatment.

The beneficial effect of nutrient on the rooting of Norway spruce cuttings has been reported by the authors (7, 14); a complete nutrient, in solution, was applied at weekly intervals. Since that method is tedious, in the experiments reported here certain nutrient chemicals were mixed with a carrier dust and applied to the cuttings in a single treatment. Potassium phosphate was chosen since phosphates are usually associated with root growth; potassium is an absolutely essential element, and all meristematic tissues are rich with it (26). However, the effect of this chemical seemed to be concerned with the development of buds rather than the initiation of roots. Likewise, when cuttings of Japanese yew (*Taxus cuspidata* Sieb. and Zucc.) were treated with a dust containing complete nutrient the main effects were on the buds (16). An increase in new growth was noticed in the original experiment with nutrient watering (7).

Both ethyl mercuric phosphate and ethyl mercuric bromide, as disinfectant chemicals, have been used but no essential differences in their effects have been noted. The purpose of these chemicals was to protect the bases of the cuttings from fungi (8). However, it turned out that the effects were concerned with new growth. In previous work (17) mercury influenced rooting favourably to a small extent. In other experiments (10, 14) mercury reduced rooting.

Sugar is thought to supply the cutting with energy during the period prior to root formation. Its effects were not great in Experiment 2, and in previous work (10, 14, 17) the results were variable.

The results of treatments with various chemicals do not indicate any essential differences between the effects of phytohormone chemicals and the others. This would seem to corroborate the opinion recently expressed by Swingle (31) that in dealing with root-forming substances the effects of materials foreign to the plant are being studied.

The detrimental effects of waxing are in agreement with a recent report on the effect of the same wax emulsion on white pine cuttings (2).

Success in vegetative propagation of conifers appears to relate to conditions of propagation, type of cuttings, and seasonal variations of the parent plant, rather than to any chemical treatments. Elucidation of the physicochemical bases for the association of these factors must await further experimental study.

References

1. COCHRAN, W. G. Empire J. Exptl. Agr. 6 : 157-175. 1938.
2. DEUBER, C. G. Trans. Conn. Acad. Arts Sci. 34 : 1-83. 1940.
3. DEUBER, C. G. and FARRAR, J. L. J. Forestry, 38 : 578-585. 1940.
4. FARRAR, J. L. Forestry Chron. 15 : 152-163. 1939.
5. FARRAR, J. L. and GRACE, N. H. Can. J. Research, C, 18 : 612. 1940.
6. GRACE, N. H. Can. J. Research, C, 17 : 178-180. 1939.
7. GRACE, N. H. Can. J. Research, C, 17 : 312-316. 1939.
8. GRACE, N. H. Can. J. Research, C, 17 : 321-333. 1939.
9. GRACE, N. H. Can. J. Research, C, 17 : 376-379. 1939.
10. GRACE, N. H. Can. J. Research, C, 18 : 13-17. 1940.
11. GRACE, N. H. Can. J. Research, C, 18 : 457-468. 1940.
12. GRACE, N. H. Can. J. Research, C, 19 : 40-41. 1941.
13. GRACE, N. H. Can. J. Research, C, 19 : 99-105. 1941.
14. GRACE, N. H. and FARRAR, J. L. Can. J. Research, C, 18 : 401-414. 1940.
15. GRACE, N. H. and FARRAR, J. L. Can. J. Research, C, 18 : 591-598. 1940.
16. GRACE, N. H. and FARRAR, J. L. Can. J. Research, C, 19 : 21-26. 1941.
17. GRACE, N. H., FARRAR, J. L., and HOPKINS, J. W. Can. J. Research, C, 18 : 566-577. 1940.
18. GRACE, N. H. and THISTLE, M. W. Can. J. Research, C, 18 : 122-128. 1940.
19. HITCHCOCK, A. E. and ZIMMERMAN, P. W. Contrib. Boyce Thompson Inst. 10 : 461-480. 1939.
20. HITCHCOCK, A. E. and ZIMMERMAN, P. W. Contrib. Boyce Thompson Inst. 11 : 143-160. 1940.
21. HUBERT, B., RAPPAPORT, J., and BEKE, A. Mededeelingen Landbouwhoogeschool Opzoekingsstations Gent, 7 : 1-103. 1939.
22. JACOBS, M. R. Australia Forestry Bureau Bull. 25. 1939.
23. KIRKPATRICK, H., JR. Florists' Exchange, 92 (14) : 13-18. 1939.
24. KIRKPATRICK, H., JR. Am. Nurseryman, 71 (8) : 9-12. 1940.
25. KOMISSAROV, D. A. Compt. rend. acad. sci. U.R.S.S. (n.s.) 21 (9) : 453-456. 1938.
26. MILLER, E. C. Plant physiology. 2nd ed. McGraw-Hill Book Company, Inc., New York and London. 1938.
27. RAPPAPORT, J. Mededeelingen Landbouwhoogeschool Opzoekingsstations Gent, 7 : 291-360. 1939.
28. SNEDECOR, G. W. Statistical Methods. Collegiate Press, Inc., Ames, Ia. 1937.
29. SNOW, A. G., JR. U.S. Dept. Agr. Northeast. Forest Exptl. Sta. Occasional Paper 11. 1940.
30. STOUTEMYER, V. T. Proc. Am. Soc. Hort. Sci. 36 : 817-822. 1938.
31. SWINGLE, C. F. Botan. Rev. 6 (7) : 301-355. 1940.
32. THIMANN, K. V. and DELISLE, A. L. J. Arnold Arboretum, 20 (1) : 116-136. 1939.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 19, SEC. D. JULY, 1941 NUMBER 7

STORAGE STUDIES ON LIQUID BLOOD[1]

BY M. W. THISTLE[2], N. E. GIBBONS[3], W. H. COOK[2], AND C. B. STEWART[4]

Abstract

The effect of the addition of glucose, storage temperature, exclusion of air, and constant rotation on the haemolysis of human blood was investigated. Added glucose had the most marked inhibitory effect of any of the factors studied. Addition of glucose results in a pH drift to the acid side; the extent of this drift is governed by storage temperature. Buffering of blood mixtures indicates that the beneficial effect of glucose is only partially dependent upon the pH changes produced. Storage temperature affected also the rate of haemolysis. Within the temperature range −4° to 12.5° C., minimum haemolysis occurred between 2.5° and 6° C. Excluding air and keeping the blood in slow motion also had highly significant effects in the reduction of haemolysis during the first four weeks in storage.

Other factors studied less extensively included differences between donors, dilutions, container sizes, and air pressures. Donor differences are important when the rate of haemolysis is low, but at higher rates they are masked by treatment effects. Dilution with isotonic solutions retards haemolysis markedly. Haemolysis occurred to the same extent in small and large containers. The shape of the container and its position during storage had no demonstrable effect. Preliminary storage tests at pressures of 0 to 350 mm. of mercury above atmospheric pressure indicated that storage at the normal blood pressure may be of some value in reducing haemolysis. Cell volume changes appear to depend on storage temperature.

Introduction

An investigation to determine the best storage temperature for liquid blood was referred to us by the Subcommittee on Blood Storage of the Associate Committee on Medical Research, since unique cold storage facilities were available in the laboratories devoted to food storage and transport investigations. It is generally stated and accepted that blood kept at room temperature haemolyzes more rapidly than blood kept at lower temperatures, but experimental evidence on this point appears to be limited (5). In practice blood is usually stored in the region of 0° to 6° C. (1, 2, 3). The temperature range from −4° to 12.5° C. was studied in this investigation.

Although the primary object was a study of storage temperature, several other factors were investigated. This made it possible to assess not only the effect of storage temperature when other conditions varied, but also the

[1] *Manuscript received April 24, 1941.*

Contribution from the Division of Biology and Agriculture of the National Research Council, Ottawa. N.R.C. No. 999.

With some financial support from the Subcommittee on Blood Storage of the Associate Committee on Medical Research.

[2] *Biochemist, Food Storage and Transport Investigations.*

[3] *Bacteriologist, Food Storage and Transport Investigations.*

[4] *Formerly Assistant Secretary, Associate Committee on Medical Research, now Flight Lieutenant, R.C.A.F. Medical Branch.*

influence of temperature in relation to the effect of other factors. The choice of conditions other than temperature was determined largely by an effort to approximate certain body conditions. Accordingly the blood was kept in motion, air was excluded, glucose added, and finally pressure applied. Portions of this work were confirmatory in nature; Rous and Turner (10) demonstrated that glucose inhibited haemolysis; DeGowin, Harris, and Plass (4) recently reported "the remarkable inhibition of haemolysis under anaerobic conditions".

Materials and Methods

The effect of the various treatments on the quality of the blood was determined by measuring the degree of haemolysis. Although this criterion is commonly used for estimating the suitability of blood for transfusion, it is recognized that it may not be entirely adequate. Nevertheless, haemolysis is one of the changes that renders stored blood unfit for transfusion, and since it can be measured accurately and quickly, it is well suited to routine studies of this sort.

The number of cells and their fragility were determined in the early experiments but were later abandoned as these two criteria were too insensitive to distinguish between many of the treatments. Measurements of total and oxyhaemoglobin, cell volume, and pH were made on all samples. Sterility tests were also made on the majority of the samples.

Preliminary experiments were made with sheep's blood but the resistance of the sheep cell to haemolysis made it unsuitable for the present studies. Even after 12 weeks' storage the haemolysis was less than 0.5%. Sheep's blood was also difficult to sample and analyse accurately owing to the formation of clots during storage. Human blood was therefore used in subsequent experiments.

Blood was drawn from healthy male adults in amounts of 200 to 300 ml. in flasks containing the appropriate solutions. The resulting blood mixture was then apportioned directly into storage tubes of about 10 ml. capacity. Aseptic precautions were taken at all stages until the blood was removed from storage. In most of the experiments samples were withdrawn from storage at weekly intervals.

On removal from storage the cells were resuspended by careful shaking, and samples were removed for haemoglobin, cell fragility, and sterility tests. The remainder of the sample was centrifuged under standard conditions and the cell volume estimated. The plasma was then removed for analysis, and pH measurements made on the remaining materials, principally red cells, with a glass electrode.

The degree of haemolysis and the amount of total and oxyhaemoglobin were determined with an Evelyn photoelectric colorimeter (6). Analyses of whole blood (plus anticoagulant) were made after dilution to 0.2%. The haemoglobin in the supernatant plasma (haemolysis) was measured after dilution to 50 or 10%, according to the degree of haemolysis. These dilutions

insured that all observations were made in the central range of the colorimeter scale where errors are minimal. With the small samples available this procedure necessitated in certain instances the use of the "micro" attachment for the Evelyn instrument. When the colour was determined, using the 540 filter employed for oxyhaemoglobin, the plasma of freshly drawn blood always showed detectable absorption. This absorption could not be demonstrated with certainty in plasma dilutions comparable to those made with whole blood.

TABLE I

PLASMA COLOUR AFTER ONE DAY'S STORAGE CALCULATED AS MG. HAEMOGLOBIN PER 100 ML.

All samples rolled with glucose in completely filled tubes. Single observations.

Donors	Storage temperature, °C.			Mean
	0	5	10	
G.P.	41	34	38	38
A.L.	39	41	43	41
E.C.	72	57	66	65
R.M.	72	72	81	75
Mean	56	51	57	55

Table I shows that the plasma colour after one day's storage, calculated as haemoglobin, differs for different donors. Certain information indicated that part of this plasma colour might be due to dissolved haemoglobin, and, lacking definite evidence to the contrary, it was expressed as haemolysis. Initial variations of this sort, as well as variations in the haemoglobin contents of the blood due to donor differences and differential dilution, all affect the degree of haemolysis if it is expressed as a percentage of the total haemoglobin present. In these circumstances it seemed desirable to express the haemolysis directly as the mg. dissolved haemoglobin per 100 ml. of blood. In any event the statistical design of all these experiments prevented the initial colour or concentration of haemoglobin from being confused with treatment effects.

Although it would be desirable to maintain the same sodium citrate concentration, isotonicity, and dilution in all samples, this becomes impossible if glucose is to be added to certain samples and not to others. When glucose is added the tonicity or dilution must be changed. The results of some initial experiments on the effects of container shape and size, reported later, showed that dilution with isotonic solutions caused a marked decrease in haemolysis during storage. Since this tended to increase the storage period required to distinguish between the treatments under investigation, an attempt was made to maintain the same dilution and final sodium citrate concentration, and accept a somewhat hypertonic solution. Some workers have found less haemolysis following the use of hypertonic diluents (9). When glucose was not added, nine volumes of blood were added to one volume of 3.8% (isotonic)

anhydrous sodium citrate. The final samples were therefore isotonic and contained 0.38% citrate. When required, the necessary amount of a 50% glucose solution, sterilized by filtration, was added to this citrated mixture to make the final mixture 2.8% glucose. Since the volume of added glucose was small, the dilution and citrate concentration were not changed appreciably, and the final osmotic pressure corresponded to 1.5 times that of an isotonic solution. Before adding the 50% glucose solution the cells were allowed to settle and the glucose solution added to the supernatant plasma. This procedure caused no detectable haemolysis, whereas the direct addition of concentrated glucose haemolyzed some of the cells.

During storage the blood was held under conditions that maintained the reported temperature well within $\pm 0.1°$ C. in the majority of instances. In some tests, however, the temperature occasionally fluctuated as much as $\pm 0.4°$ C. Some blood samples were kept in motion during storage; this was accomplished by rotating the tubes about their horizontal axis at a rate that kept the cells in suspension (3 rev. per hr.). Stationary tubes were kept in a vertical position, since the container experiments showed no differences due to the position of the tubes. Tubes to be completely filled were drawn down at one end and provided with a short length of rubber tubing. After they were filled and stoppered aseptically, they were inverted, the retained air bubble was trapped in the tubing, and the tubing clamped off next to the tube. The partly filled tubes had an air space of 3 to 4 ml. between the 10 ml. of blood and the stopper. According to DeGowin, Harris, and Plass (5) blood in tubes stoppered in this way behaves similarly to blood stored in tubes plugged with cotton.

Results

Container Experiments

Prior to the main part of the investigation some work was undertaken to obtain information on the effect of the shape of the storage vessel, shaking, air space in the container during storage, and subsequent aeration. The initial experiments were made with citrated bovine blood held in 300 ml. containers. Containers of two shapes were used, Florence flasks and tubes 1.5 in. in diameter and 12 to 14 in. long. In half the containers about 10% of the volume was air space whereas the other half were completely filled. Duplicate sets of these vessels were held in both horizontal and vertical positions during storage and shaking. As soon as the blood had reached the storage temperature (0° C.), the samples to be shaken were oscillated at 116 cycles per min. for five hours and, after two weeks' storage, were given a similar treatment. At the end of the storage period the samples were heated to 37° C. and two litres of air bubbled through the blood for a period of one hour.

The results of haemolysis measurements made at each stage are reported in Table II and a statistical analysis of these in Table III. Completely filling the containers reduced haemolysis significantly. Shaking, particularly after storage, and aeration increased haemolysis, presumably owing to increased

cell fragility. Indications are that the differences due to the shape of the container and its position during storage are insignificant.

Later experiments on the effect of container size were rather unsatisfactory owing to the fact that the bovine blood used clotted when stored for longer than two weeks. For this reason the experiments were repeated (300 ml. compared with 10 ml.), using human blood diluted (4 to 15) with isotonic dextrose and citrate in the following proportions: blood, 4 parts; dextrose, 10 parts; citrate, 1 part. The storage chamber was held at about 3.0° C.

TABLE II

HAEMOLYSIS OF BOVINE BLOOD AS AFFECTED BY CONTAINER AND TREATMENT, MG. PER 100 ML.

	Shape and position			Average
	Round	Long vertical	Long horizontal	
Free space	45*	48	54	49.0
No free space	42	44	40	42.0
Average	43.5	46.0	47.0	45.5

	Stage				Average
	Storage for 3 days	Storage for 14 days	Storage for 14 days plus 5 hours' additional shaking	Storage for 15 days plus aeration	
Standing	28**	38	—†	60	41.0
Shaken	24	38	58	79	49.8
Average	26.0	38.0	48.0	69.5	45.4

* *Figures represent means of eight analyses.*
** *Figures represent means of six analyses.*
† *Value carried over from preceding stage.*

TABLE III

ANALYSIS OF VARIANCE OF THE HAEMOLYSIS OF BOVINE BLOOD AS AFFECTED BY CONTAINER AND TREATMENT

Source of variance	Degrees of freedom	Mean square
Shape	2	47
Space	1	488*
Stage	3	4121**
Shaking	1	892**
Shape × space	2	154
Stage × shaking	3	489**
Error (Residual)	32	66

* *Surpasses 5% level of significance.*
** *Surpasses 1% level of significance.*

A table of means and the corresponding analysis of variance is presented in Table IV. The efficacy of dilution in reducing haemolysis (8) is shown by the fact that after seven and one-half weeks' storage, the haemolysis of these samples, corrected for dilution differences, is comparable with that developed after two weeks' storage in concentrated samples (Fig. 1). The main point of interest is that no difference due to the size of the container can be demonstrated. The absence of free space was slightly beneficial, but not significantly so. The long storage period reversed the beneficial effect of rolling over shorter periods, reported later, presumably because of increased cell fragility. The main difference emerging from this experiment was that between donors.

TABLE IV

HAEMOLYSIS AS AFFECTED BY TREATMENT

Treatment means

Source of variance	Haemolysis, mg. per 100 ml. Means of eight observations
Large container, 300 ml.	42
Small container, 10 ml.	43
Completely filled tubes	41
Free space	44
Rolling	47
Standing	38
Donor F.S.	49
Donor T.S.	36

Analysis of variance

Source of variance	Degrees of freedom	Mean square
Container size	1	3.1
Free space	1	33.1
Rolling	1	351.6**
Donors	1	637.6**
Error (Residual)	11	23.7

** *Surpasses 1% level of significance.*

Cell Counts

Typical red and white cell counts obtained after various treatments and storage conditions appear in Table V. There was considerable variation in the number of red cells in the same blood from time to time, probably owing to difficulties of mixing and sampling. Indeed, these variations were larger

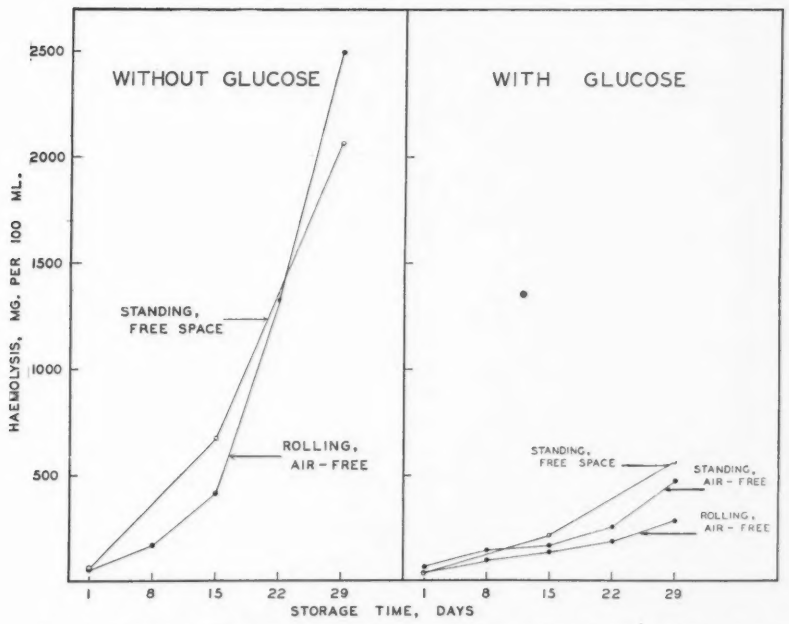


FIG. 1. *Successive reductions in haemolysis obtained by adding glucose, excluding air, and keeping the blood in motion.*

than were required to account for the maximum haemolysis observed colorimetrically. Since there was no definite trend, it is concluded that none of the treatments or storage conditions caused any significant change in the number of red cells.

The results in Table V show that the number of white cells decreased under all storage conditions tested. This decrease was greatest at the low storage temperatures, and least at 10° C., the highest temperature employed for this test. Glucose appears to accelerate the destruction of white cells.

Cell Fragility

There was a definite increase in cell fragility under all conditions of storage (Table V). Cells stored at 5° C. were the least, and those held at 10° C. the most fragile. Storage at 0° C. resulted in an intermediate fragility. Other storage temperatures were not included in these tests. When glucose was added the cells were more fragile during the first two weeks of storage, but after that the fragility of blood cells stored with and without glucose appeared to be about the same. During this period no differences in cell fragility could be detected between samples kept in motion and stationary samples.

TABLE V

CHANGES IN CELL COUNT AND CELL FRAGILITY DURING STORAGE

Donor	Temp., °C.	Time, days	R.B.C. (millions)	W.B.C. (thousands)	Fragility
		Glucose, stationary, completely filled containers			
G.P.	10	1	5.40	4.25	0.60 – 0.55*
		3	5.12	4.10	.60 – .55
		8	4.68	3.75	.65 – .60
		15	4.73	3.75	.70 – .65
		22	5.52	2.25	.85 – .75
	5	1	4.48	5.05	.65 – .60
		3	4.96	4.75	.65 – .60
		8	4.19	2.40	.65 – .60
		15	4.55	1.20	.65 – .60
		22	4.86	1.05	.80 – .70
	0	1	4.84	4.50	.65 – .60
		3	5.30	3.00	.65 – .60
		8	4.10	1.75	.70 – .60
		15	4.32	.15	.70 – .60
		22	4.65	.05	.80 – .70
		No glucose, rolled, completely filled containers			
A.L.	10	1	6.40	5.50	.45 – .40
		2	6.00	6.00	.45 – .40
		4	5.20	5.00	.50 – .45
		8	5.20	4.90	.55 – .50
		29	5.18	4.00	— – —
	5	1	4.24	5.10	.45 – .40
		2	5.13	6.20	.45 – .40
		8	4.30	2.25	.50 – .45
		Glucose, rolled, completely filled containers			
A.L.	10	1	6.87	7.50	.60 – .50
		2	5.82	6.40	.60 – .55
		8	5.37	3.80	.65 – .60
		29	5.28	2.15	
	5	1	6.62	6.25	.60 – .50
		2	5.48	6.00	.60 – .55
		8	4.84	1.60	.60 – .55

* *First figure is the highest concentration of saline in which partial haemolysis occurred. Second figure is highest concentration in which haemolysis was complete.*

R.B.C. = *Red blood cells.*

W.B.C. = *White blood cells.*

TABLE VI

OXYHAEMOGLOBIN, GM. PER 100 ML.

All samples rolled with glucose in completely filled tubes. Means of four observations.

Storage time, days	Temperature, °C.			Average
	0	5	10	
1	13.6	13.3	13.4	13.5
8	13.5	13.5	13.3	13.4
15	13.4	13.0	12.8	13.1
22	13.2	12.9	12.7	12.9
29	13.2	12.8	12.6	12.8
Average	13.4	13.1	13.0	13.15

Total and Oxyhaemoglobin

There was some time-to-time variability in the total and oxyhaemoglobin contents of the blood from the same donor. These were generally small and attributable in part to difficulties of mixing and analysing the stored blood. Nevertheless, the means show some tendency for the haemoglobin to decrease during storage, particularly at the higher storage temperatures, and these decrements (Table VI) usually attained statistical significance. Since the contents of total and oxyhaemoglobin were closely correlated in most samples, only the results for the oxyhaemoglobin are reported in Table VI. These measurements were made simultaneously with those for haemolysis, and were taken from samples kept in motion during storage since the errors of sampling were least for such material.

Haemolysis

The first experiment on storage temperature contrasted the effects of 0° and 5° C. over periods up to 31 days. Glucose was added and both air free and partly filled tubes were represented. Samples stored in the stationary condition were compared with others kept in motion by rotation.

The average values for haemolysis, as mg. per 100 ml., for each condition appear in Table VII. The main points of interest were: that less haemolysis occurred at 5° C., and that both exclusion of air and rotation during storage reduced the degree of haemolysis. The statistical analysis given in the second part of Table VII shows that these differences were statistically significant.

These results led to a more extensive study, subsequently referred to as the main experiment, involving the blood from six donors. This experiment was designed to permit comparison of the following conditions: presence or absence of glucose, moving and stationary storage, and partly and completely filled tubes, in addition to the effects of storage time, storage temperature, and donor differences. The results of this study appear in Figs. 1, 2, 3, and Tables VIII, IX, and X.

TABLE VII

HAEMOLYSIS AS AFFECTED BY TREATMENT. DONOR A.M.

Treatment means

Source of variance	Haemolysis, mg. per 100 ml.
	Means of 12 observations
Completely filled tubes	240
Free space	287
Rolling	238
Standing	289
5° C.	229
0° C.	298
	Means of 8 observations
Storage time, 10 days	167
24 days	287
31 days	337

Analysis of variance

Source of variance	Degrees of freedom	Mean square
Temperature	1	29,191**
Free space	1	13,301**
Rolling	1	16,172**
Storage time	2	60,654**
Error (Residual)	17	1147

** *Surpasses 1% level of significance.*

TABLE VIII

HAEMOLYSIS, MG. PER 100 ML.

Means of three observations. All samples standing

Temp., °C.	Donor M.K. Free space		Donor A.W. Glucose	
	Glucose	No glucose	Completely filled tubes	Free space
0	288	671	292	335
2.5	233	692	176	272
5.0	248	907	195	245
7.5	263	1195	201	248
10.0	330	1193	234	251

Means of five observations. All samples in completely filled tubes

Temp., ° C.	Donor A.L. Rolled		Donor E.C. Glucose		Donor G.P. Glucose	
	Glucose	No glucose	Rolled	Standing	Rolled	Standing
0	172	826	208	213	158	184
5.0	121	680	145	204	116	148
10.0	197	1167	199	239	174	217

TABLE IX

HAEMOLYSIS AS AFFECTED BY DONOR DIFFERENCES

All samples with glucose. Means of 15 observations

Conditions	Date	Donors	Haemolysis, mg. per 100 ml.	Average of pairs
Standing with free space	22/5/40	A.W. M.K.	270 272	271
Rolled in completely filled tubes	13/5/40	A.L. E.C.	163 184	174
	31/5/40	G.P. R.M.	150 156	153

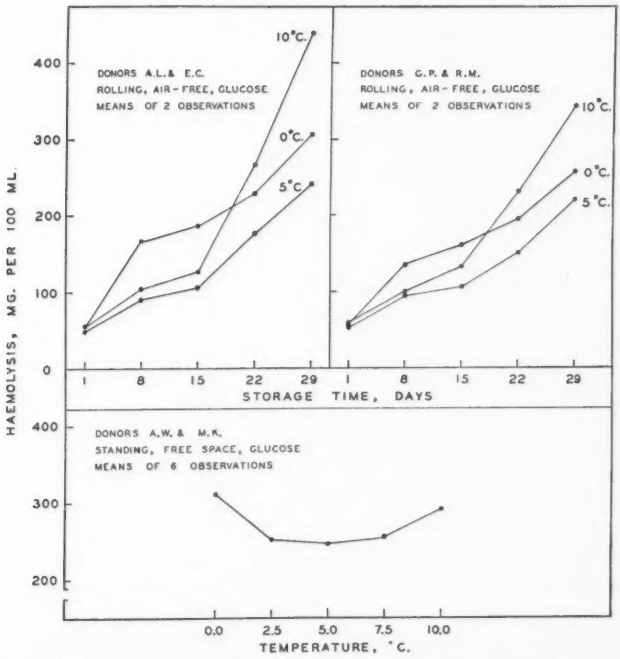


FIG. 2. *Effects of storage temperature upon haemolysis, showing differential response with storage time.*

In general there was a gradual increase in haemolysis with time in storage. The rate of haemolysis, however, is materially reduced (Fig. 1) by simulating certain body conditions during storage, e.g., addition of glucose, completely filling the containers, and keeping the blood in slow motion. In the absence of glucose (Fig. 1), haemolysis is also reduced by completely filling the con-

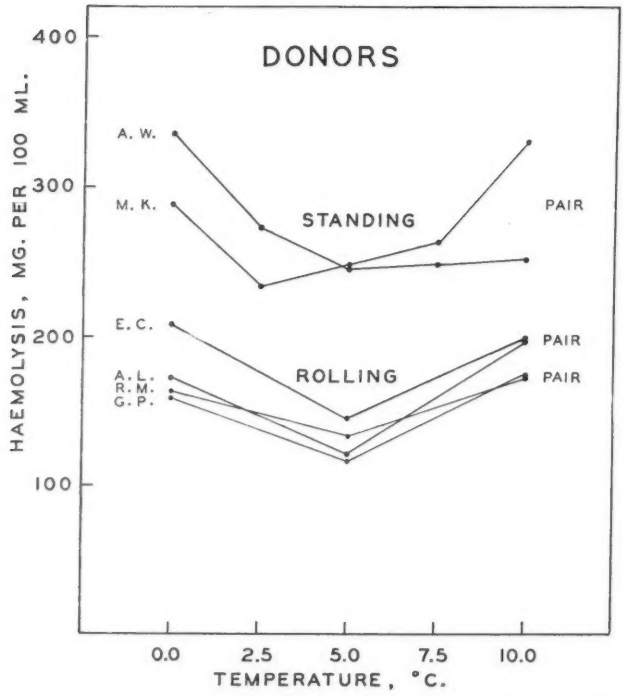


FIG. 3. *Donor differences with respect to haemolysis. The standing pair show differential response with storage temperatures.*

tainers, and keeping the blood in motion, during the early part of the storage history. On long storage, however, considerable damage is evident, indicating that the mechanical action of rolling may injure fragile cells unprotected by glucose.

The curve in the lower section of Fig. 2 represents an attempt to distinguish between five storage temperatures at 2.5° C. intervals. An analysis of variance failed to distinguish between them. Other tests of significance showed only that 0° C. was inferior to the remaining temperatures. The effect of storage temperature and the differential effects of storage time at different temperatures are shown in the upper section of Fig. 2. Analysis of the results obtained at 0°, 5°, and 10° C., i.e., over 5° C. intervals, showed that 5° C. was significantly better than the 0° and 10° C. storage temperatures in the duplicate tests conducted (Tables VIII and X).

Differences between donors are shown in Tables IX and X*(d)*. The differential effects of temperature on the haemolysis of blood from different donors are shown in Fig. 3. Since the blood samples from this group of donors were treated in pairs, it is possible to obtain a strict comparison

TABLE X(*a*)

ANALYSIS OF VARIANCE OF HAEMOLYSIS AS AFFECTED BY TREATMENT, WITH SPECIAL REFERENCE TO THE EFFECTS OF KEEPING THE BLOOD IN MOTION

All samples with glucose in completely filled tubes

Source of variance	Degrees of freedom	Mean square	
		Donor E.C.	Donor G.P.
Rolling	1	9083**	8602**
Storage time	4	102176**	75907**
Temperature	2	5626**	10168**
Storage × temperature	8	4599**	3342**
Rolling × storage	4	5495**	2322**
Rolling × temperature	2	1876**	200
Error (Residual)	8	220	143

** *Surpasses 1% level of significance.*

TABLE X(*b*)

ANALYSIS OF VARIANCE OF HAEMOLYSIS AS AFFECTED BY TREATMENT, WITH SPECIAL REFERENCE TO THE INFLUENCE OF GLUCOSE

Source of variance	Samples rolled in completely filled tubes		Samples standing with free space	
	Donor A.L.		Donor M.K.	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Glucose	1	3971968**	1	3261722**
Storage time	4	1932138**	2	4190465**
Temperature	2	204154	4	115158
Storage × temperature	8	92156	8	72247
Glucose × storage	4	1263119**	2	1413434**
Glucose × temperature	2	115556	4	87207
Error (Residual)	8	55762	8	44306

** *Surpasses 1% level of significance.*

between donors by comparing the samples that were treated identically throughout. Tables IX and X(*d*) show that the difference between donors A.L. and E.C. is significant, but that the differences within the remaining pairs failed to attain significance. Table IX also shows a difference between pairs treated identically, but collected at different times. In practice this difference is the one most commonly observed. The variation in the initial colour of the plasma from different donors (Table I) bears little relation to the rates of haemolysis obtained after treatment and storage. It will be recalled, however, that in the container study (Table IV), where the rate of haemolysis was rather low, the difference between donors was the greatest difference observed.

TABLE X(*c*)

ANALYSIS OF VARIANCE OF HAEMOLYSIS AS AFFECTED BY TREATMENT, WITH SPECIAL REFERENCE TO THE EFFECTS OF COMPLETELY FILLING THE TUBES

All samples standing with glucose. Donor A.W.

Source of variance	Degrees of freedom	Mean square
Free space	1	19001**
Storage time	2	536914**
Temperature	4	9396**
Storage × temperature	8	4119*
Free space × storage	2	17188**
Free space × temperature	4	1232
Error (Residual)	8	897

* *Surpasses 5% level of significance.*
** *Surpasses 1% level of significance.*

TABLE X(*d*)

ANALYSIS OF VARIANCE OF HAEMOLYSIS AS AFFECTED BY TREATMENT, WITH SPECIAL REFERENCE TO DONOR DIFFERENCES

Source of variance	Samples rolled with glucose in completely filled tubes				Samples standing with glucose and free space	
	Degrees of freedom	Mean square			Degrees of freedom	Mean square
		Donors A.L. and E.C.	Donors A.L. and G.P.	Donors G.P. and R.M.	Donors A.W. and M.K.	
Donors	1	3286**	1360	327	1	39
Storage time	4	68536**	63199**	41322**	2	735616**
Temperature	2	12702**	11728**	6308**	4	4876
Storage × temperature	8	4650**	4210**	1826*	8	4536
Donors × storage	4	130	517	995	2	2772
Donors × temperature	2	724	222	213	4	3834
Error (Residual)	8	259	346	364	8	1997

* *Surpasses 5% level of significance.*
** *Surpasses 1% level of significance.*

The next experiment was designed primarily to secure further information on storage temperature. Storage temperatures were −4.0°, −2.0°, 0°, 2.5°, 5.0°, 6.0°, 7.5°, 10.0°, and 12.5° C. Glucose was added to give isotonic and slightly hypertonic solutions, since it was felt that a slightly hypertonic mixture might be advantageous. The blood in isotonic and hypertonic glucose was prepared by mixing eight volumes of blood in one volume of isotonic citrate, with one volume of 5.4 or 8.1% glucose, resulting in final

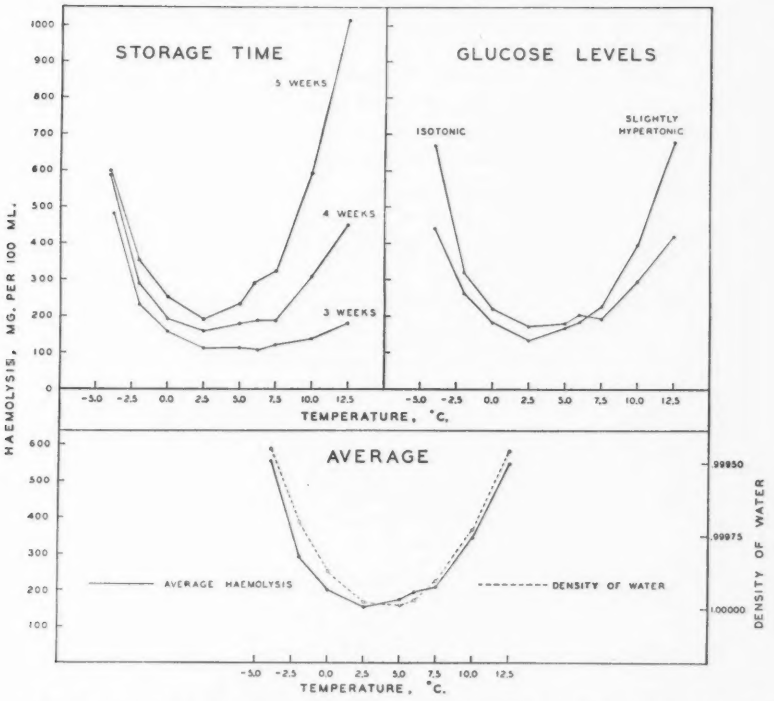


FIG. 4. *Haemolysis as affected by storage temperature. The effects of storage time and differing levels of glucose are shown in the component parts of the parabola.*

glucose concentrations of 0.54 and 0.81%, respectively. All of the tubes were completely filled and were kept in motion since these conditions had been shown to be effective in reducing haemolysis.

The effect of altering the glucose level of the solution is evident from Fig. 4. Over all temperatures there is no demonstrable effect. However, the slightly hypertonic solution appears to be more effective for reducing haemolysis at the lower range of temperatures studied, whereas the isotonic blood kept better at the higher temperatures.

These results, obtained in approximately isotonic concentration, cannot be compared directly with hypertonic samples in the main experiment (1.5 times the osmotic pressure of an isotonic solution), since the dilution differed in the two series. Average values for a number of comparable storage and other conditions in the two series indicate that the haemolysis for the isotonic and hypertonic mixtures, after proportionality corrections for dilution, was 202 and 255 mg. haemoglobin per 100 ml., respectively. Although this suggests that the isotonic solution is superior, this is believed to be due primarily to the greater dilution used in the isotonic mixture. It has been shown earlier

that the degree of haemolysis decreases with increasing dilution, but this factor was not studied quantitatively.

At temperatures of $-4.0°$ C. and $-2.0°$ C. duplicate samples were stored in both the frozen and supercooled states. The frozen samples were always 80 to 100% haemolyzed; all subsequent figures and discussion of the results obtained at subzero temperatures therefore refer to the supercooled samples.

The influence of storage temperatures is shown by the parabolic curve in Fig. 4, representing the average haemolysis for all storage periods and glucose levels. This curve passes through a long minimum between about $2.5°$ and $6.0°$ C.

The influence of storage time is also shown in Fig. 4, by curves obtained for the results after three, four, and five weeks' storage. These curves show that haemolysis accelerates at the higher storage temperatures, confirming the results of experiments previously reported.

The parabolic curve relating haemolysis and storage temperature (Fig. 4) closely approximates the density–temperature curve for water, the principal constituent of blood. The slope of the upper curves in Fig. 4, however, shows that the apparent association is largely fortuitous.

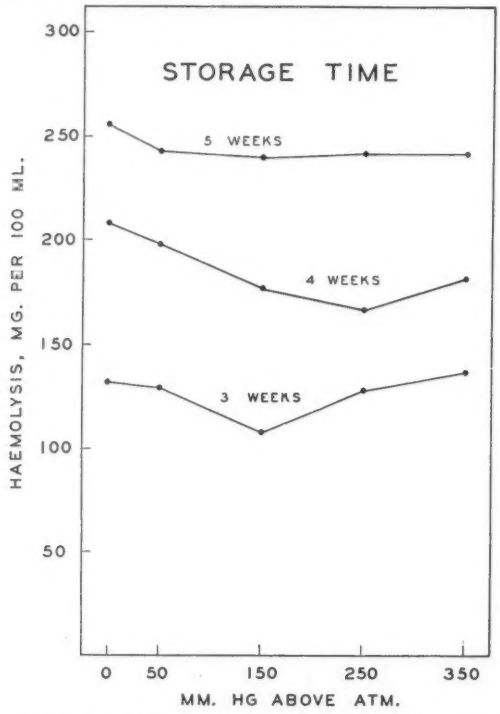


FIG. 5. *The effects of additional pressure upon haemolysis.*

Effects of Pressure on Haemolysis

A preliminary experiment to investigate the effects of pressure upon haemolysis was undertaken near the end of the investigation. Three samples were put under pressure at each of the following levels: atmospheric pressure and 50, 150, 250, and 350 mm. of mercury above atmospheric pressure. For pressure variations due to leakage, which were less than 10 mm., compensation was made by the addition of the necessary amount of mercury in the manometer side-arms. The system was inspected twice daily, and after a few days all leaks were stopped. The room temperature was kept at about 3.0° C.

The results are presented in Fig. 5. One sample at each pressure was removed after three, four, and five weeks' storage. After three weeks the value nearest to blood pressure in the body, 150 mm. of mercury, showed a saving of about 20% of the haemolysis produced under atmospheric con-

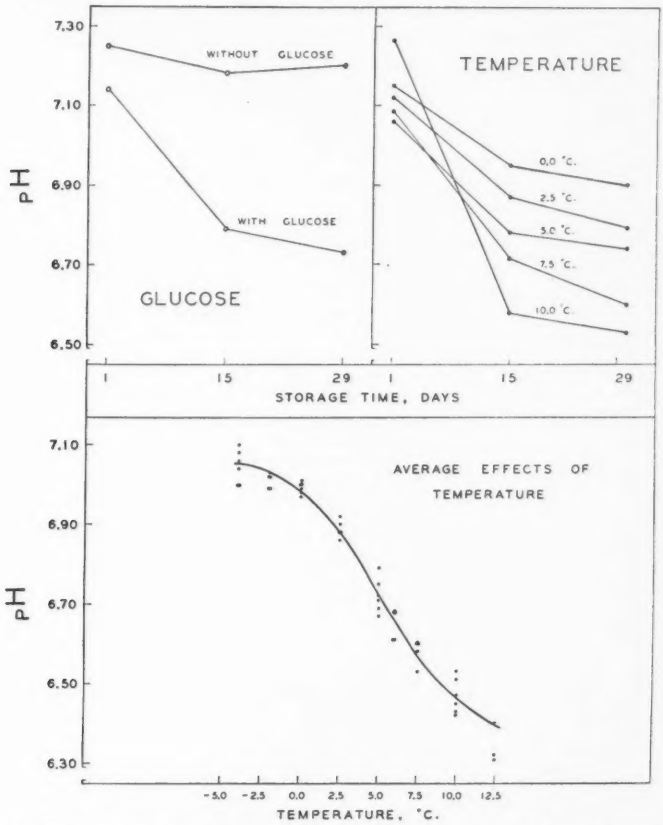


FIG. 6. *The pH drift when glucose is added, showing that the extent of drift is dependent upon storage temperature.*

ditions. After four weeks, 150 and 250 mm. of mercury are still producing some beneficial effect, but after five weeks the saving of cells under pressure is negligible. The preliminary nature of the experiment and the small number of samples involved made it impossible to demonstrate any statistically significant effect due to pressure. Nevertheless the results are interesting, and suggest that a study on the effects of storing blood under a pressure comparable with that in the body might be of some value.

Changes in pH during Storage

The results of pH measurements made on the samples in the main experiment showed that blood stored without glucose showed no significant pH changes. The addition of glucose, however, causes the pH to drift to the acid side. The extent of this change was greatest at the high temperatures and decreased regularly as the temperature decreased (Fig. 6, upper section). These observations were confirmed by the results of later experiments shown in Fig. 6 (lower section). Minor differences due to storage practice and glucose levels, as well as error, contribute to the scattering of the points. The curve fitted to the points graphically indicates not only that the pH change is greatest at the higher storage temperatures, but also that the slope of this pH–temperature curve is greatest in the temperature range where minimum haemolysis occurs.

These results suggested the use of buffered blood mixtures. Isotonic mixtures of mono- and dibasic sodium phosphate (NaH_2PO_4 and Na_2HPO_4) were used to maintain the pH at higher and lower levels than that of normal blood. In addition to the untreated controls, samples were prepared with

TABLE XI

CHANGES IN pH AND HAEMOLYSIS OF BUFFERED BLOOD SOLUTIONS

pH changes, means of three observations

Storage time, days	Buffered above normal pH	Unbuffered (normal behaviour)	Buffered below normal pH
0	7.50	7.32	6.50
12	7.30	7.04	6.60
24	7.27	6.96	6.62
36	7.14	6.85	6.59

Haemolysis, mg. per 100 ml., means of three observations

Glucose level	Buffered above normal pH	Unbuffered (normal behaviour)	Buffered below normal pH
No glucose	756	2551	366
Isotonic glucose	88	88	295
Hypertonic glucose	101	88	268

added glucose in isotonic and slightly hypertonic solutions to represent the condition of changing pH during storage. All samples were stored at 5° C.

The results of the pH measurements appear in Table XI. It can be seen that the pH of the buffered samples changed somewhat during storage, but always remained above or below the pH of unbuffered blood.

The results of haemolysis measurements on these samples are also shown in Table XI. As expected, the presence of glucose markedly inhibits haemolysis. In the absence of glucose, buffering is effective in reducing haemolysis, particularly on the acid side of the normal pH. When glucose is added buffering on the acid side causes increased haemolysis, whereas buffering on the alkaline side of the normal value has little or no effect. The two glucose levels employed appeared to have little influence on the haemolysis.

From these results it appears that the protective action of glucose is not primarily the result of the pH changes it produces during storage. The addition of glucose rather than buffering on the acid side would appear to be the best protection against haemolysis.

Cell Volume

In general the swelling of the erythrocytes on storage paralleled the pH changes, following temperature in the same manner (Fig. 7). The decrease in cell volume at the end of the storage period for the higher temperatures can

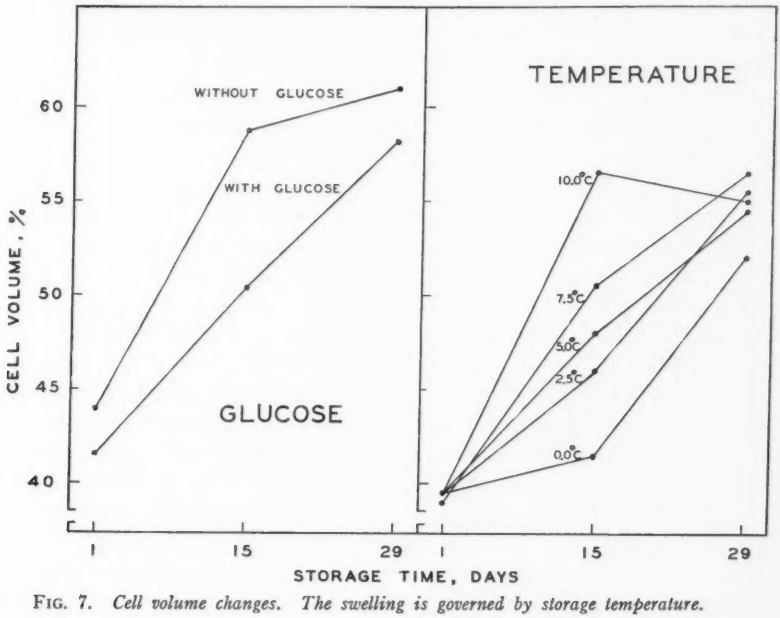


FIG. 7. *Cell volume changes. The swelling is governed by storage temperature.*

be explained by cell rupture, as is apparent from the greater haemolysis at these temperatures. However, the cells appear to swell faster in the absence of glucose (Fig. 7).

Summary and Conclusions

Minimum haemolysis occurs in the temperature region of 2.5° to 6.0°C. Differences in haemolysis within this range are rather small. A storage temperature of about 4.0° C. might be suggested as the mid-point of the minimum haemolysis range. A differential temperature effect is noted, such that the rate of haemolysis accelerates with time to a greater degree at the higher temperatures studied than at the lower temperatures.

The addition of glucose inhibits haemolysis to a marked extent. Also, the addition of glucose results in a pH drift to the acid side during storage. In general the higher the storage temperature, the greater is the pH drift. Change in pH is most sensitive to temperature in the temperature region associated with minimum haemolysis. Buffering of blood mixtures inhibits haemolysis to some extent when glucose is absent, but in the presence of glucose buffering is either harmful or of no effect, depending on the pH level. This indicates that the beneficial effect of glucose is at best only partially dependent on the pH changes produced.

Keeping the blood in motion in the presence of glucose inhibits haemolysis for the first four weeks of storage. In citrate alone rolling is beneficial for the first two weeks. It would appear to be likely, from the work of Fåhræus (7), that the efficacy of rolling in reducing haemolysis is due to the maintenance of the cell–plasma interface.

Storing the blood in completely filled tubes inhibits haemolysis for the first four weeks of storage.

Some information was obtained on a number of other factors which, however, were not studied extensively. Of those studied, donor differences appeared to be relatively unimportant in comparison with treatment effects when the rate of haemolysis is comparatively rapid. However, when the rate of haemolysis is rather slow, donor differences are an important factor. Dilution with glucose–citrate mixtures in isotonic concentration causes a marked reduction in haemolysis. Over the entire temperature range, no difference in haemolysis could be attributed to slight alterations in glucose concentration. However, there is some evidence that blood, if made slightly hypertonic with glucose, will keep better at the lower temperatures studied. This finding is in line with other investigations (9). A preliminary experiment on the effects of pressure on haemolysis suggests that storing blood under a pressure comparable with that in the body might be of some value.

No difference in haemolysis can be detected when containers of practical dimensions are compared with the sample containers used in this investigation.

Acknowledgments

Grateful acknowledgment is made to the Red Cross Society Transfusion Service, especially to Mr. J. R. Potter, the Secretary, who made arrangements for the medical examinations, and to Dr. S. F. Service and the members of the staff of the Ottawa Civic Hospital who made the examinations. We also wish to acknowledge the assistance of Drs. Rooke and Caldwell, who collected the samples from human donors, and to thank Dr. Paterson, of the Ottawa Civic Hospital, for making their services available.

Mr. A. M. Adams, Laboratory Assistant, was largely responsible for testing sterility, and for making many cell counts and fragility tests. Mr. A. J. McLeod, Laboratory Assistant, also gave valuable technical aid in several phases of the work. Messrs. T. A. Steeves and A. E. Chadderton, of the Biology Division, were of great service in constructing the necessary equipment. Our thanks are also due to the members of the staff of the National Research Council, whose contributions of blood have made the work possible.

References

1. ANON. Brit. Med. J. 1 : 1187-1188. 1939.
2. ANON. J. Am. Med. Assoc. 113 (21) : 1893. 1939.
3. ANON. J. Am. Med. Assoc. 113 (23) : 2061-2062. 1939.
4. DEGOWIN, E. L., HARRIS, J. E., and PLASS, E. D. Proc. Soc. Exptl. Biol. Med. 40 (1) : 126-128. 1939.
5. DEGOWIN, E. L., HARRIS, J. E., and PLASS, E. D. J. Am. Med. Assoc. 114 (10) : 850-855. 1940.
6. EVELYN, K. A. J. Biol. Chem. 115 (1) : 63-75. 1936.
7. FÅHRÆUS, R. Lancet, 237 (2) : 630-635. 1939.
8. HARINGTON, C. R. and MILES, A. A. Brit. Med. J. 1 : 1202-1203. 1939.
9. MACQUAIDE, D. H. G. and MOLLISON, P. L. Brit. Med. J. 2 : 555-556. 1940.
10. ROUS, P. and TURNER, J. B. J. Exptl. Med. 23 : 219-238. 1916.

THE ABILITY OF SHEEP'S ERYTHROCYTES TO SURVIVE FREEZING[1]

By A. H. Woodcock[2], M. W. Thistle[3], W. H. Cook[3], and N. E. Gibbons[4]

Abstract

Erythrocytes from sheep's blood were subjected to several prefreezing treatments, frozen at various rates, and stored at temperatures from −2° C. to −190° C. Minimum haemolysis generally occurred when the cells were suspended in 1 to 1.5 isotonic glucose and frozen extremely rapidly in liquid air. During subsequent storage about two-thirds of the cells survived 32 days' storage at −190° C., only about half survived at −78° C., and haemolysis was practically complete at higher temperatures. Cells dried from the frozen state, *in vacuo*, appeared normal, but always haemolyzed when liquids were added. Cells held in both the supercooled and partly frozen condition at temperatures slightly below the freezing point showed that least haemolysis occurred in the supercooled samples. In the frozen samples the extent of haemolysis increased as the temperature decreased and was practically complete at −8° C. Although the results show that the formation of ice crystals is one of the primary causes of cell disintegration, differences between animals and other unknown factors also have a significant influence on the ability of the cells to withstand freezing.

Introduction

If whole blood or suspensions of red cells in isotonic solutions are frozen, the erythrocytes are practically all destroyed. This has, up to the present time, precluded the use of freezing temperatures for the storage of blood for transfusion or other uses. The object of the present study was to determine the effect of certain pretreatments, rates of freezing, and subsequent holding temperature on the degree of haemolysis of frozen blood.

The results of recent investigations (5, 3) indicate that the formation of ice crystals is a cause of death when living organisms are exposed to freezing temperatures. Whether erythrocytes are considered dead or alive, it may reasonably be assumed that crystal formation is responsible for most of the destructive influence caused by freezing. Consequently, freezing procedures that minimize crystal formation should enable some proportion of red cells to survive.

Extremely rapid freezing rates may pass the material through the temperature range in which rapid crystal formation occurs so quickly that few crystal nuclei are formed. In such a system the water will be present in the vitrified state. Here the practical considerations are whether a suspension of erythrocytes can be frozen at a sufficiently rapid rate, and whether crystal formation in the vitrified mass can be avoided at practical storage temperatures

[1] *Manuscript received April 24, 1941.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. N.R.C. No. 1000.

[2] *Biophysicist, National Research Laboratories.*

[3] *Biochemist, National Research Laboratories.*

[4] *Bacteriologist, National Research Laboratories.*

over sufficient periods. In this connection it appears (1, 3, 4) that the extent of crystal formation varies directly with the proportion of water to solids in the test material.

Crystal formation can also be reduced by the use of storage temperatures only slightly below the freezing point of the solution. Since blood has a freezing point of about $-0.6°$ C., whereas the eutectic temperature either is, or could be adjusted to, a much lower value, it follows that the extent of crystal formation depends on the storage temperature. This suggests the possibility of using storage temperatures only slightly below the freezing point. These considerations also indicate that very slow freezing might be beneficial. The crystals produced between the freezing point and the eutectic will be pure water. If these crystals form in the suspending medium, as distinct from the cells, they may not be destructive, and the gradual reduction in the water content of the cells may depress their freezing point sufficiently to avoid freezing or favour vitrification. The experiments were designed to examine all of these possibilities.

Materials

All of these experiments were made with citrated sheep's blood. It was intended that these should be preliminary to similar studies on human blood. Since the results of other investigations (6) have indicated that the erythrocytes from sheep were much more resistant to haemolytic influences than those from humans, it represented better material for differentiating between the various treatments.

Methods

The degree of haemolysis was determined with an Evelyn photoelectric colorimeter (2) and, where necessary, the condition of surviving cells was determined by microscopic examination. Since the oxyhaemoglobin content of the test solutions varied considerably, the haemolysis is subsequently expressed as a percentage of the oxyhaemoglobin present.

Methods for increasing the proportion of solids to water included evaporation and addition of glucose. Preliminary studies showed that blood could be partly dried by evaporation from Visking casings at room temperature. By this procedure a 50% loss of weight was accompanied by no appreciable haemolysis. Glucose in concentrations up to 15% produced little or no haemolysis, but at higher concentrations haemolysis increased rapidly.

Results

Rapid Freezing in Liquid Air

In the first tests a small wire loop was immersed successively in the blood preparation, liquid air, and a thawing solution. Whole blood and blood concentrated by partial drying were thawed in unconcentrated plasma. The results indicated that a 25% moisture loss was the best of these treatments, about 50 to 60% of the cells surviving. Blood cells were also suspended, frozen, and thawed in glucose solutions of various tonicities. The best glucose

concentrations appeared to be between 5.4 and 8.1% (1 and 1.5 isotonic); in these about 75% of the cells survived.

In the next experiments a fine metal screen was immersed in the blood preparation, drained, immersed in liquid air, and stored. The excess liquid air was allowed to boil off at the storage temperature. Samples were stored at −190° C. (liquid air), −78° C. (solid carbon dioxide), −40° C., and −18° C. The samples were removed after varying periods up to 32 days, thawed by immersion in glucose solutions, and analysed. Blood preparations included whole blood, blood dried to 13 and 30% loss of weight, and cells washed and suspended in 5, 10, 15, 20, 25, and 30% glucose solutions.

The haemolysis resulting from each treatment averaged over all storage treatments and periods is given in Table I. These results show that freezing and thawing in hypertonic glucose solutions is generally better than the other treatments but, on the average, only about 30% of the cells survived.

TABLE I

EFFECT OF FREEZING MEDIUM ON SURVIVAL

Freezing medium	Cells in glucose solutions, %						Whole blood	13% dried	30% dried
	5	10	15	20	25	30			
Average haemolysis, %	72	65	68	68	63	69	74	72.5	72.5

Table II shows the effect of temperature and time of storage averaged over all treatments, and Table III, the results of a statistical analysis of the data for the three lowest temperatures and storage periods up to 18 days. The first analyses were made after one day's storage, and haemolysis indicated at that time includes any that occurred during freezing and thawing as well as storage. Storage over longer periods does cause some additional haemolysis, but storage temperature seems to be the most important factor. At temperatures of −40° C., or higher, the cells were almost completely haemolyzed,

TABLE II

EFFECT OF STORAGE TIME AND TEMPERATURE ON HAEMOLYSIS OF STORED BLOOD, %

Storage period, days	Storage temperature, °C.			
	−190°	−78°	−40°	−18°
1	36.4	41.0	85.0	100
4	37.7	48.0	91.4	100
8	37.2	45.0	95.4	97.5
18	(69.0)	(68.5)	99.0	—
32	35.1	46.0	100.0	—

Note: Values in parentheses doubtful.

at −78° C. about half the cells survived, and at −190° C. nearly two-thirds were intact at the end of 32 days' storage.

TABLE III

ANALYSIS OF VARIANCE OF RESULTS† OF HAEMOLYSIS DURING FREEZING AND STORAGE

Source of variance	Degrees of freedom	Mean square
Time	3	3043***
Temperature	2	24,666***
Freezing medium	8	420*
Error	93	176

* *Denotes 5% level of significance.*
*** *Denotes 0.1% level of significance.*
† *Results at −18° C. and those for 32 days' storage not used in this analysis.*

From these results it appears that liquid air temperatures would be required to preserve blood in the frozen state. This was considered impracticable, and further investigations designed to reduce the haemolysis observed after one day's storage and attributable in part to the freezing and thawing procedures were abandoned.

Drying from the Frozen State

Since frozen blood cannot be preserved at practicable temperatures, an attempt was made to dry the frozen samples under high vacuum. The blood was prepared as previously described, immersed in a liquid air container, and the entire unit sealed up and the liquid air pumped off in a room at −40° C. Following removal of the liquid air, the water evaporated from the sample under high vacuum and condensed on a series of finned surfaces cooled with liquid air.

Blood cells that had been completely dried in this way appeared normal when examined microscopically. However, all attempts to resuspend these cells in liquid media of various sorts at various rates failed. In these circumstances this line of attack was abandoned.

Partial Freezing

Attention was turned to the storage of partly frozen and supercooled blood at temperatures ranging from −2° to −8° C. Samples (2-ml.) of a mixture of 5 ml. citrated sheep's blood and 20 ml. of 8 or 12% glucose solution were used. All the samples were allowed to attain the desired storage temperatures in controlled baths. One-half of the samples were then seeded with a small ice crystal and the other half allowed to remain in the supercooled state. Below −4° C. the supercooled samples were so unstable that crystallization occurred during storage. Samples were removed for analysis after 1, 8, and 18 days' storage. The results showed that haemolysis increased with time,

the average for the seeded samples being 21.7, 54.0, and 54.0%, and for the supercooled 7.6, 9.3, and 9.5% for 1, 8, and 18 days' storage, respectively. When the results are averaged for all storage periods, it is evident from Table IV that more haemolysis occurred in 12 than in 8% glucose; that haemolysis increased as the storage temperature decreased; and that the degree of haemolysis was usually less in the supercooled than in the frozen samples held at the same temperature. Although these results show that the number of surviving cells decreases as the extent of crystal formation increases, they do not indicate that partial freezing could become a practicable method for preserving suspensions of red cells. Supercooling at temperatures down to −4° C. is better than freezing in the same range, but other studies (6) have shown that storage in the supercooled state causes more haemolysis than storage at about 5° C.

TABLE IV

HAEMOLYSIS OF SUPERCOOLED AND PARTLY FROZEN CELL SUSPENSIONS

Temp., °C.	State	Haemolysis, %	
		8% glucose	12% glucose
−2	Partially frozen	2.8	7.5
−4	Partially frozen	26.1	38.0
−5	Partially frozen	36.0	56.0
−6.5	Partially frozen	55.0	69.7
−8	Partially frozen	59.3	81.8
−2	Supercooled	1.7	16.2
−4	Supercooled	3.3	16.2

Slow Freezing to −40° C.

The results of the experiments described above indicated that the freezing and thawing of blood was accompanied by considerable haemolysis, and even if this could be avoided, considerable difficulty would be experienced in providing the requisite liquid air temperatures for storage. Nevertheless, in order to complete the study, some experiments were undertaken on the effect of freezing rate. In this connection it seemed desirable to cool the samples to a sufficiently low temperature to assure complete freezing and avoid other complications. A final temperature of −40° C. was chosen.

A preliminary experiment showed distinct promise. A 2-ml. sample frozen in air at −40° C. and thawed at room temperature showed only 20% haemolysis. In later experiments the rate of freezing these 2-ml. samples was varied by plunging some tubes in liquid air, then removing them to a room at −40° C., and by placing others in a large volume of ethylene glycol (as thermal ballast) in a Dewar flask and allowing the whole to cool in a room at −40° C. The time required to cool the samples, therefore, was varied from a few seconds to 48 hr. Rates of thawing were varied by exposing some tubes to room temperature (30 min.) and exposing others in ethylene glycol baths originally

at −40° C. to room temperature (48 hr.). These experiments were uniformly unsuccessful, the haemolysis varying from 90 to 100% with no evidence as to the best procedure.

Owing to the marked discrepancy between these results and those of the preliminary experiment, another series of tests was conducted to investigate other variables. These included: a study of blood from four different sheep; effect of storage prior to freezing; and the effect of various glucose concentrations in addition to whole blood. More complete details of procedure appear in Table V, together with the results of the haemolysis measurements. The freezing and thawing procedures were uniform for all samples. Freezing was accomplished by placing 8 ml. in a test tube in a room at −40° C. and thawing by removing to room temperature for one hour. Samples containing hypertonic glucose were brought slowly back to isotonicity by allowing the required amount of water to diffuse into the solution through Visking casings at 5° C.

A statistical analysis of the results of these experiments also appears in Table V. There was a significant difference between the resistances of the erythrocytes of different sheep to these treatments, the average haemolysis

TABLE V

HAEMOLYSIS OF SHEEP'S ERYTHROCYTES FOLLOWING VARIOUS PREFREEZING TREATMENTS AND STORAGE PERIODS AT −40° C.

Prefreezing treatment	Freezing medium	Postfreezing treatment	Haemolysis, %		
			Period of frozen storage		
			1 day	8 days	28 days
1. None	Plasma	None	100	100	100
2. Held in plasma 48 hr.	Plasma	None	99	100	100
3. None	Glucose, 5.4%	None	67.5	77.8	78.5
4. Held in glucose 48 hr.	Glucose, 5.4	None	73.7	87.8	91.5
5. Held in plasma 48 hr.	Glucose, 5.4	None	61.8	70.8	80.0
6. None	Glucose, 5.4	Held 24 hr.	72.8	76.3	77.0
7. Held in glucose 48 hr.	Glucose, 5.4	Held 24 hr.	70.6	85.8	87.3
8. Held in plasma 48 hr.	Glucose, 5.4	Held 24 hr.	60.3	70.7	79.5
9. None	Glucose, 8.1	Returned to isotonicity	51.0	64.8	71.3
10. Held in glucose 48 hr.	Glucose, 8.1	Returned to isotonicity	77.3	84.5	92.5
11. Held in plasma 48 hr.	Glucose, 8.1	Returned to isotonicity	53.5	71.8	80.8

ANALYSIS OF VARIANCE OF RESULTS 6 TO 11 INCLUSIVE, GIVEN ABOVE

Source of variance	Degrees of freedom	Mean square
Concentration of glucose	1	235*
Prefreezing treatment	2	1530***
Storage period	2	1803***
Variance between sheep	3	695***
Error	40	40

* *Denotes 5% level of significance.*
*** *Denotes 0.1% level of significance.*

in glucose solutions varying from 68 to 79% for different animals. The effect of the other treatments can best be assessed from the average values obtained from the four sheep. Cells held in glucose for 48 hr. prior to freezing suffered greater haemolysis than cells frozen in glucose within three hours after collecting. On the other hand, cells stored in plasma for 48 hr. prior to freezing in glucose were usually more resistant to haemolysis than the unstored samples. Cells frozen in plasma, however, were always completely haemolyzed, demonstrating the protective action of glucose at this stage. Haemolysis also increased with increasing time of storage.

Discussion

Since it is outside the scope of this paper to consider the temperature effects of any chemical changes that might produce haemolysis, the matter is discussed from a purely physical point of view. Using the vitrification theory as a working hypothesis it appears that crystal formation produces a breakdown of the erythrocytes. As shown in Table IV minimum haemolysis occurs when the material is supercooled and there are no crystals present. Moreover, haemolysis increases, in the seeded samples, with lowering temperature which is equivalent to greater crystal formation. From the results, shown in Table II, it was suspected that freezing caused some vitrification, and when the temperature was raised recrystallization took place with consequent haemolysis. Also from Tables I and V it appears that hypertonic glucose solutions aid in preserving cells by decreasing the relative water content and inhibiting crystal formation to some degree.

References

1. Barnes, W. H. and Matthews, F. W. Biodynamica, 2 (49) : 1-7. 1939.
2. Evelyn, K. A. J. Biol. Chem. 115 (1) : 63-75. 1936.
3. Goetz, A. and Goetz, S. S. Biodynamica, 2 (43) : 1-8. 1938.
4. Lipman, C. B. Biodynamica, 2 (45) : 1-4. 1939.
5. Luyet, B. J. and Gehenio, P. M. Biodynamica, 2 (42) : 1-7. 1938.
6. Thistle, M. W., Gibbons, N. E., Cook, W. H., and Stewart, C. B. Can. J. Research, D, 19 : 185-205. 1941.

CANADIAN WILTSHIRE BACON

XX. A COMPARISON OF CERTAIN CHEMICAL AND PHYSICAL PROPERTIES OF CANADIAN AND DANISH BACONS, AND THEIR RELATION TO FLAVOUR QUALITY[1]

By W. Harold White[2], C. A. Winkler[3], and W. H. Cook[2]

Abstract

Canadian bacon generally contained more curing salts, and was slightly drier and tougher than the Danish product. Although fat was present in approximately the same proportions in the bacons of both countries, that of Canadian bacon was slightly softer. The relative variability of Canadian and Danish bacons differed with the property considered. For Canadian bacon the most important source of the observed variations was usually the differences between the product prepared by different factories, whereas for Danish bacon, differences between sides were the principal source of variation.

Of the properties studied, flavour excellence in Wiltshire bacon was dependent primarily on the absence of excessive amounts of curing salts. Other contributing factors, of secondary importance, were high moisture content, tenderness, and a low proportion of good quality fat. Improvement in the flavour quality of Canadian Wiltshire bacon could be achieved by the use of milder cures, and the general adoption of improved handling and curing practices.

Introduction

The results of extensive investigations on the relative flavour excellence of Canadian and Danish Wiltshire bacons showed a superiority of the Danish over the Canadian product (9). It was impossible, however, to obtain reliable information from these subjective tests as to the causes of superiority or inferiority of a given product because of the difficulties involved in adequately describing quality, and the impossibility of suitably assessing the significance of such comments by statistical methods. Consequently physical and chemical examinations of the bacons were made to determine the attributes of quality responsible for the superiority of the Danish product. The results of these objective measurements and their correlation with the subjective flavour scores are presented in this paper.

The selection of the measurements was based on those considered necessary to characterize the product, with particular emphasis being placed on the determinations that the comments of the tasters suggested as being important with respect to quality. The results of these analyses permitted a description of the product from the two countries with respect to the absolute values of the several properties and their variability. They also provided data that could be correlated with the subjective flavour scores obtained on the same

[1] *Manuscript received April 18, 1941.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Issued as Paper No. 65 of the Canadian Committee on Storage and Transport of Food, and as N.R.C. No. 998.

[2] *Biochemist, Food Storage and Transport Investigations.*

[3] *Formerly Biophysicist, Food Storage and Transport Investigations; now Assistant Professor of Chemistry, McGill University, Montreal, P.Q.*

material, thus giving information on the factors responsible for quality independent of the origin of the product.

Material and Methods

Since it was impossible to examine quantitatively all of the several thousand samples used in the flavour investigations, it was necessary to restrict the present studies to one group of samples. Of the material available, that from the incomplete randomized block experiment (9), in which each factory was represented by nine sides, was considered the most suitable for studying differences between and within factories. Furthermore, the results of this experiment provided reliable data for the computation of the relations between the measured properties and quality scores obtained from three independent panels.

Samples consisting of comparable portions of the prime back, directly posterior to the ribs, from nine sides selected from each of 18 Canadian and 10 Danish factories were packed in latex bags, sealed, boxed, and shipped at −12° C. (10° F.) from London, England. Upon arrival at Montreal, the boxes were packed in dry ice, and transferred immediately to the laboratories, where they were stored at −29° C. (−20° F.) until analysed. It is believed that as a result of these precautions little or no change in the measured properties occurred during shipment.

Of the several properties of bacon that might have been studied, the results of the flavour investigations suggested that the concentrations of chloride, nitrate, and nitrite, the content and quality of the fat, moisture content, and toughness would probably differ between the two countries, and correlate with the results of the flavour tests. In addition, these probably constitute the most important of the common properties of bacon, with the exception of colour.

Although all nine sides from each factory were used for the flavour tests, it was considered that fewer samples were required for the objective measurements described here, because of their greater accuracy and precision. The chloride, nitrate, and nitrite contents of five samples taken at random from the nine available for each of 18 Canadian and 10 Danish factories were determined by methods previously described (7).

The fat content was estimated by careful separation of the external layer from the lean meat, followed by weighing. Samples of three sides picked at random from each of 15 Canadian and nine Danish factories were treated in this manner.

Of the chemical methods available for estimating the quality of the fat, determination of the iodine number was considered to be most suitable (3, 5). Kaufmann's procedure (4) for this determination was used in the study of the external fat samples removed from three sides from each of 15 Canadian and nine Danish factories.

The moisture contents of five samples from each of 18 Canadian and 10 Danish factories were determined by drying *in vacuo* at 100° C. for 24 hr., or to constant weight.

Measurements of the relative toughness of samples of three sides from each of 15 Canadian and nine Danish factories were made by a method described previously (8).

Statistical methods (6) were used to reduce and interpret the data for each property. From the means, the standard deviation, and coefficient of variability between samples, it was possible to assess and compare the absolute and relative variability of the properties both within and between countries. An analysis of variance served to determine the relative importance of the possible sources of variation. The overall variability of Canadian and Danish Wiltshire bacons with respect to any one property may be divided broadly into differences in the properties of the sides within and between countries. Variations within countries may be further classified as differences between the sides produced in any one factory and differences between the product from different factories. For intercountry comparisons, differences between sides and between factories, as well as the overall difference between countries, can be distinguished. It was also of importance to assess the differences between factories and between sides within factories irrespective of country of origin.

The degree of interrelation of the properties studied with flavour score was estimated by the calculation of simple, and in some instances, partial, coefficients of correlation (6) between the mean values of the measurement and the quality score for each plant. The average values for factories rather than for countries were chosen for statistical treatment since they give a more representative evaluation of the properties studied. Although the use of the data for each side would have been still more suitable, the method of scoring for flavour, employed in the incomplete randomized block experiment, did not permit this. Correlation coefficients were calculated between the measurements and the flavour scores obtained from each of the three panels, since, although the over-all results for any one panel showed definitely that the quality of Canadian and Danish Wiltshire bacons differed, the relative order of the quality scores for plants within either country varied between the panels.

Results

Differences between Canadian and Danish Bacons

The mean values of the properties measured for Canadian and Danish Wiltshire bacons, together with analyses of variance are shown in Tables I to VII. Canadian bacon contained, on the average, significantly more sodium chloride, nitrate, and nitrite than Danish (Tables I, II, and III). The relative magnitude of these differences was especially large for nitrate, of which Canadian bacon contained approximately seven times more than the Danish samples studied. The fat of Canadian bacon also had a significantly

TABLE I

SODIUM CHLORIDE CONTENT OF CANADIAN AND DANISH WILTSHIRE BACONS

Statistic	Country	
	Canada	Denmark
Mean, %	7.47	6.21
Standard deviation, %	0.84	1.09
Coefficient of variability	11.3	17.5

ANALYSIS OF VARIANCE

Variance attributable to:	D.f.	Mean square
Between countries	1	50.8*
Between factories for both countries	26	7.31***
Between sides within factories for both countries	112	0.872
Between factories within Canada	17	9.66***
Between sides within Canada	72	0.698
Between factories within Denmark	9	2.87*
Between sides within Denmark	40	1.18

* *Indicates 5% level of significance.*
*** *Indicates 0.1% level of significance.*

TABLE II

SODIUM NITRATE CONTENT OF CANADIAN AND DANISH WILTSHIRE BACONS

Statistic	Country	
	Canada	Denmark
Mean, %	0.157	0.024
Standard deviation, %	0.028	0.015
Coefficient of variability	17.9	63.3

ANALYSIS OF VARIANCE

Variance attributable to:	D.f.	Mean square
Between countries	1	0.569**
Between factories for both countries	26	0.042***
Between sides within factories for both countries	112	0.0006
Between factories within Canada	17	0.0639***
Between sides within Canada	72	0.0008
Between factories within Denmark	9	0.0004
Between sides within Denmark	40	0.0002

** *Indicates 1% level of significance.*
*** *Indicates 0.1% level of significance.*

TABLE III

SODIUM NITRITE CONTENT OF CANADIAN AND DANISH WILTSHIRE BACONS

Statistic	Country	
	Canada	Denmark
Mean, p.p.m.	74	34
Standard deviation, p.p.m.	62	18
Coefficient of variability	85.1	51.3

ANALYSIS OF VARIANCE

Variance attributable to:	D.f.	Mean square
Between countries	1	49,504**
Between factories for both countries	26	6083**
Between sides within factories for both countries	112	2630
Between factories within Canada	17	8892**
Between sides within Canada	72	3921
Between factories within Denmark	9	778*
Between sides within Denmark	40	312

* *Indicates 5% level of significance.*
** *Indicates 1% level of significance.*

higher mean iodine number (Table VII). Bacon from both countries had approximately the same fat to lean ratio, namely 60 : 40 (Table VI). Although the average moisture content of Danish bacon was slightly higher, the difference failed to reach the level of statistical significance (Table IV).

TABLE IV

MOISTURE CONTENT OF CANADIAN AND DANISH WILTSHIRE BACONS

Statistic	Country	
	Canada	Denmark
Mean, %	59.6	61.1
Standard deviation, %	5.78	6.06
Coefficient of variability	9.69	9.93

ANALYSIS OF VARIANCE

Variance attributable to:	D.f.	Mean square
Between countries	1	133
Between factories for both countries	26	93.1***
Between sides within factories for both countries	112	34.6
Between factories within Canada	17	106**
Between sides within Canada	72	33.4
Between factories within Denmark	9	69.5*
Between sides within Denmark	40	36.8

* *Indicates 5% level of significance.*
** *Indicates 1% level of significance.*
*** *Indicates 0.1% level of significance.*

Canadian bacon was, on the whole, slightly tougher, but not significantly so, than the Danish product (Table V). The comments of the judges who made the subjective tests would suggest that definite differences in toughness did exist between Canadian and Danish bacons. That this was not confirmed here may be due to the fact that the objective measurements were made on uncooked material. It has been shown that toughness measurements should be made preferably on cooked samples (2).

TABLE V

TOUGHNESS OF CANADIAN AND DANISH WILTSHIRE BACONS

Statistic	Country	
	Canada	Denmark
Mean, work units	2.40	2.09
Standard deviation, work units	1.13	0.903
Coefficient of variability	46.9	43.2

ANALYSIS OF VARIANCE

Variance attributable to:	D.f.	Mean square
Between countries	1	3.18
Between factories for both countries	22	1.53
Between sides within factories for both countries	48	0.908
Between factories within Canada	14	1.83
Between sides within Canada	30	1.27**[1]
Between factories within Denmark	8	0.985
Between sides within Denmark	18	0.816**[1]

** *Indicates 1% level of significance.*

[1] *Exceeds 1% level of significance when compared with the mean square attributable to experimental error.*

Differences in the properties of sides from different factories regardless of country of origin significantly exceeded variations in sides within the same factories for the sodium chloride, nitrate, nitrite, and moisture contents. This suggests that these properties are to some extent a function of factory practice. Similar comparisons for toughness (Table V), fat content (Table VI), and the iodine number of the fat (Table VII) were not significant.

It was also of interest to determine the major source of variability in the sides from each of the two countries. Such a comparison for Canadian bacon showed that differences between sides originating from different factories significantly exceeded variations within these same factories for the sodium chloride, nitrate, and nitrite contents. Similar comparisons for Danish bacon showed significant differences in the sodium chloride and nitrite contents. Both Canadian and Danish sides showed significant variations in toughness, indicating that tenderness, as determined here, was an inherent property of the side.

TABLE VI

FAT CONTENT OF CANADIAN AND DANISH WILTSHIRE BACONS

Statistic	Country	
	Canada	Denmark
Mean, %	58.9	59.5
Standard deviation, %	6.06	6.13
Coefficient of variability	10.3	10.3

ANALYSIS OF VARIANCE

Variance attributable to:	D.f.	Mean square
Between countries	1	6.22
Between factories for both countries	22	34.4
Between sides within factories for both countries	48	37.0
Between factories within Canada	14	37.7
Between sides within Canada	30	36.7
Between factories within Denmark	8	28.6
Between sides within Denmark	18	37.6

TABLE VII

IODINE NUMBER OF FAT OF CANADIAN AND DANISH WILTSHIRE BACONS

Statistic	Country	
	Canada	Denmark
Mean	55.6	52.1
Standard deviation	4.04	3.76
Coefficient of variability	7.26	7.23

ANALYSIS OF VARIANCE

Variance attributable to:	D.f.	Mean square
Between countries	1	214**
Between factories for both countries	22	11.7
Between sides within factories for both countries	48	15.5
Between factories within Canada	14	17.1
Between sides within Canada	30	16.3
Between factories within Denmark	8	2.34
Between sides within Denmark	18	14.2

** *Indicates 1% level of significance.*

Although similar treatments for the other properties failed to reach the level of statistical significance, it is of interest to note that for Canadian bacon the variance attributable to differences between factories was usually greater than that for differences between sides within factories, whereas for the Danish product the more important source of variance was between sides.

Interrelation of Objective Measurements with Flavour Scores

The values for the coefficients of correlation between the content of curing salts and quality score are shown in Table VIII. The concentration of sodium

TABLE VIII

SIMPLE AND PARTIAL COEFFICIENTS OF CORRELATION BETWEEN CHLORIDE, NITRATE, AND NITRITE CONTENT OF CANADIAN AND DANISH WILTSHIRE BACONS AND FLAVOUR SCORE

Quantities correlated	Smithfield jury test		Importers' jury test		Family group test	
	D.f.	r	D.f.	r	D.f.	r
Flavour score with:						
Content of sodium chloride	26	−0.61**	26	−0.47*	26	−0.51**
Content of sodium nitrate	26	−0.27	26	−0.39*	26	−0.44*
Content of sodium nitrite	26	−0.31	26	−0.40*	26	−0.34
Total salt content	26	−0.61**	26	−0.49**	26	−0.53**
Content of sodium nitrate independent of sodium chloride	25	−0.12	25	−0.30	25	−0.36
Content of sodium nitrite independent of sodium chloride	25	+0.03	25	−0.20	25	−0.09

* *Indicates 5% level of significance.*
** *Indicates 1% level of significance.*

chloride was significantly and negatively related to the score for all three panels. The correlations with nitrate were negative for each, and reached the level of statistical significance for the Importers' and family group panels. Nitrite was also negatively related to quality score for all three panels, but significantly so only for the Importers' panel. Since negative relations were obtained in all instances, it is implied that the eating quality is adversely affected when excessive quantities of the curing salts are present. The fact that the relations observed for nitrate or nitrite were significant for one panel and not for another may be merely fortuitous, or may be a reflection of the ability of a particular panel to distinguish between the flavour imparted by the three salts.

To determine the effect of concentration of nitrate or nitrite on flavour quality independently of chloride, which was present in much greater concentration, partial coefficients of correlation were calculated. These were negative in most instances, but failed to reach the level of statistical significance. This suggested that the most important factor was the quantity, rather than the kind, of curing salt. To test this, simple coefficients of correlation were calculated between quality score and total concentration of

salts, i.e., sum of nitrate, nitrite, and chloride. These were negative, highly significant, and, in two instances, greater than those obtained for chloride alone, although not significantly so. This indicates that the flavour quality decreased if excessive amounts of curing salts were present, regardless of the nature of the salt. However, there is some indication that chloride, present in much larger quantities, may mask the effect of nitrate and nitrite on eating quality.

Since, as has been shown previously, the mean concentrations of all three salts were greater in Canadian than in Danish bacon, the quality of Canadian bacon could be improved by the use of milder cures, in conjunction with suitable supplementary methods of preservation if necessary.

TABLE IX

SIMPLE COEFFICIENTS OF CORRELATION BETWEEN MOISTURE CONTENT AND TOUGHNESS OF CANADIAN AND DANISH WILTSHIRE BACONS AND FLAVOUR SCORE

Quantities correlated	Smithfield jury test		Importers' jury test		Family group test	
	D.f.	r	D.f.	r	D.f.	r
Flavour score with:						
Per cent moisture calculated on basis of:						
Whole meat	26	0.30	26	0.43*	26	0.42*
Salt-free meat	26	0.04	26	0.24	26	0.21
Toughness	22	−0.20	22	0.26	22	−0.19

* *Indicates 5% level of significance.*

Simple coefficients of correlation between moisture content, as calculated on a whole meat basis and quality score, were significant and positive for the Importers' panel and family group tests (Table IX). This implies that good quality in bacon is associated with a high moisture content. However, since the moisture content is to some extent dependent on the concentrations of salts present, the observed relations might merely be a reflection of the effect of salt concentration. This was tested by calculating the moisture contents on a salt-free basis, and subsequently correlating these values with the quality scores. These values were positive for each panel, but failed to reach the level of statistical significance. This would suggest that there was no relation between the moisture content of the bacon and its quality as determined here.

The coefficients obtained in comparisons of toughness with quality score were in most instances negative, but not statistically significant (Table IX). As would be expected, toughness adversely affected quality, but was apparently of less importance than the content of curing salts.

The correlations between quality score and the measurements made on the fat are shown in Table X. The coefficients for the quality of the fat, as

estimated by the iodine number, were for the most part negative, but statistically significant for the Smithfield panel alone. This indicates that the over-all flavour quality of Wiltshire bacon decreased with an increase in iodine number, i.e., a decrease in fat quality. It may be that the more unsaturated the fat, the less stable it is during cooking, with the consequent enhanced formation of products unpleasant to the taste.

TABLE X

SIMPLE COEFFICIENTS OF CORRELATION BETWEEN CONTENT AND IODINE NUMBER OF FAT OF CANADIAN AND DANISH WILTSHIRE BACONS AND FLAVOUR SCORE

Quantities correlated	Smithfield jury test		Importers' jury test		Family group test	
	D.f.	r	D.f.	r	D.f.	r
Flavour score with:						
Content of fat	22	−0.01	22	0.22	22	−0.14
Iodine number of fat	22	−0.49*	22	0.05	22	−0.14

* *Indicates 5% level of significance.*

The content of fat was for the most part negatively, but not significantly, related to the quality score. This would imply that an excess amount of fat is detrimental to quality.

Discussion

Of the physical and chemical properties of Wiltshire bacon considered here as bearing a possible relation to quality, the content of curing salts was the most important. Although the observed variations cannot be attributed entirely to this factor alone, it must be recognized that the quality of Canadian Wiltshire bacon could be improved by the use of milder cures.

As a whole the data show that the quality of the fat of Canadian Wiltshire bacon sides is satisfactory (3). However, in some instances the fat would be classed as definitely soft. In addition, it is evident that a more uniform selection and grading of sides both within and between Canadian factories exporting Wiltshire bacon would be desirable.

However, with the exception of the content of curing salts, Canadian bacon compares favourably with Danish for the properties studied here. In most instances the bacon of Canadian manufacture was more variable in nature than that originating from Denmark. For Canadian bacon the most important source of the observed variations was between sides originating from different factories. The major source in the Danish product lay between the individual sides. This suggests that considerable uniformity in factory and curing practices exists in Denmark, whereas in Canada, although each factory prepares a relatively uniform product, undesirable variability exists between the various companies exporting Wiltshire bacon. This is in agreement with the results of the subjective tests on flavour quality.

The relative variability of Canadian and Danish factories for each of the properties studied here can be seen readily from the series of histograms shown in Fig. 1. In their preparation the class interval was made approximately equal to the necessary difference for statistical significance computed for the variance between sides within factories irrespective of the country of origin (1). In this way the observed mean values for factories are distributed over the number of classes that can be distinguished experimentally from one another. It is to be noted, however, that individual results in adjacent classes may not differ significantly. Since these are self-explanatory, further discussion of them is unnecessary.

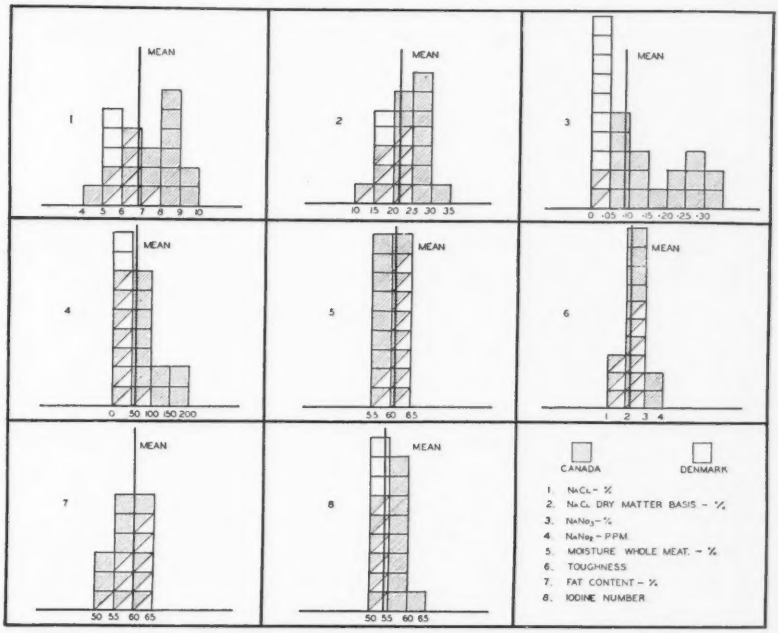


FIG. 1. *Histograms for the measured properties of Canadian and Danish Wiltshire bacons.*

The concentrations of chloride in the samples studied here were much higher than those previously obtained in 1938 for Canadian Wiltshire bacon (1). At that time it was found that the mean chloride content on a moisture- and salt-free basis was 18.2%, whereas here the mean for the same factories on a similar basis was 22.7%. These differences were found to be statistically significant.

The observed increase is presumably due either to the use of stronger curing solutions or to differences in sampling position studied in the two years. The samples examined in 1938 were from the ham, whereas those in 1939 were

removed from the back. Although it has been shown previously that significant differences with position exist even within one distinct portion of the side (1), it is doubtful if the magnitude of these normal variations in concentration is sufficiently great to account for the differences observed here.

Since significant difference in the concentrations of chloride existed between the two years, it is to be expected that the moisture content would also differ. It was found that the mean moisture content, calculated on a salt-free basis, of the samples studied here was approximately 65% as compared to a value of 73%, obtained in 1938, for samples from the same factories, which were stored and smoked on an experimental basis. Since the 1938 samples consisted of small cuts and were probably exposed to an atmosphere of lower relative humidity prior to analysis, the moisture content of these might be expected to be lower than those of 1939. An explanation of this apparent anomaly may be that British smoking practices cause excessive drying of the sides. This may possibly account for the criticism of "hardness" sometimes levelled at Canadian bacon.

Acknowledgments

The authors wish to express their appreciation to Messrs. E. G. Blake and E. A. Rooke, Laboratory Assistants, and to Dr. J. W. Hopkins, Statistician, National Research Laboratories, for their able assistance.

References

1. Cook, W. H. and White, W. H. Can. J. Research, D, 18 : 135-148. 1940.
2. Ewell, A. W. Refrig. Eng. 39 (4) : 237-240. 1940.
3. Hankins, O. G. and Ellis, N. R. U.S. Dept. Agr. Bull. 1407. 1926.
4. Kaufmann, H. P. Studien auf dem Fettgebiet. Verlag Chemie, G.M.B.H., Berlin. 1935.
5. Medwedtschuk, P. I. Biochem. Z. 214 : 282-309. 1929.
6. Snedecor, G. W. Statistical methods. Rev. ed. Collegiate Press, Inc., Ames, Ia. 1938.
7. White, W. H. Can. J. Research, D, 17 : 125-136. 1939.
8. Winkler, C. A. Can. J. Research, D, 17 : 8-14. 1939.
9. Winkler, C. A. and Cook, W. H. Can. J. Research, D, 19 : 157-176. 1941.

CANADIAN JOURNAL OF RESEARCH

Notes on the Preparation of Copy

General:—Manuscripts should be typewritten, double spaced, and the original and one copy submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulae, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. All pages should be numbered.

Abstract:—An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.

Illustrations:—Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only. Paper ruled in green, yellow, or red should not be used. The principal co-ordinate lines should be ruled in India ink and all lines should be of sufficient thickness to reproduce well. Lettering and numerals should be of such size that they will not be less than one millimetre in height when reproduced in a cut three inches wide. If means for neat lettering are not available, lettering should be indicated in pencil only. All experimental points should be carefully drawn with instruments.

Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. Small photographs should be mounted on cardboard and those to be reproduced in groups should be so arranged and mounted. The author's name, title of paper, and figure number should be written on the back of each illustration. Captions should not be written on the illustrations, but typed on a separate page of the manuscript.

Tables:—Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum.

References should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper. The form of literature citation should be that used in this Journal and titles of papers should not be given. All citations should be checked with the original articles.

The *Canadian Journal of Research* conforms in general with the practice outlined in the *Canadian Government Editorial Style Manual*, published by the Department of Public Printing and Stationery, Ottawa.